THE ROLE OF OXIDATIVE AND NITROSATIVE STRESS IN THE DEVELOPMENT OF CIRCULATORY CHANGES IN CIRRHOSIS

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ABSTRACT

There is abundant evidence that liver disease is associated with both increased production of reactive oxygen species and of nitric oxide. Previous studies of antioxidant therapy in advanced liver disease have, however, shown only minimal effects on disease progression.

In this thesis, oxidative stress is shown to be increased in patients with liver disease by quantification of plasma and urinary F_2-isoprostanes. The F_2-isoprostanes, in addition to being markers of oxidant stress, are also vasoactive compounds. This is confirmed by the demonstration that 8-isoprostaglandin $F_{2\alpha}$ causes intrahepatic vasoconstriction in rat livers, an effect which is markedly enhanced in rats with secondary biliary cirrhosis, when compared to controls. Ethanol is also shown to cause marked vasoconstriction in cirrhotic rats, though this appears to be mediated by endothelins rather than isoprostanes.

The effects of antioxidant therapy on both disease progression and haemodynamic parameters were studied. In bile duct ligated rats chronic administration of the antioxidant lipoic acid prevents the development of the hyperdynamic circulation, in association with a reduced level of nitric oxide synthase activity when compared to control rats. There is, however, no effect on either histological or biochemical parameters.

The combination of increased nitric oxide production in the presence of reactive oxygen species alters the chemistry and physiology of nitric oxide. Overproduction of a group of long acting nitric oxide carrier molecules, the S-nitrosothiols, would be predicted under such circumstances. In order to examine the biochemistry of S-nitrosothiols the development of a novel assay is described, capable of measuring plasma concentrations of these compounds down to a concentration of 5 nM. Using this assay normal plasma concentrations were shown to be orders of magnitude lower than has been previously suggested.

ABSTRACT

New insights into the chemistry of S-nitrosothiols, in particular their generation by nitric oxide under aerobic conditions, and their degradation by thiols through transnitrosation reactions is described. This development has opened the way to future studies of the role of these compounds in all disease processes including those involving the liver.

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CHAPTER – 1 -INTRODUCTION

1.1 DEFINITIONS

1.1.1 Oxidative and Nitrosative Stress

Oxidative stress can be simply defined as a disturbance in the prooxidant – antioxidant balance in favour of the former. Chemical species capable of causing oxidative stress are formed as partially reduced metabolites of molecular oxygen. They include compounds such as hydrogen peroxide and superoxide (O_2^-), which are constantly formed as a result of cellular metabolism in mitochondria and peroxisomes as well as by enzyme systems such as the cytochrome P450 enzymes, membrane associated oxidases, lipooxygenase, and xanthine oxidase. Hydrogen peroxide, though a weaker oxidising agent than superoxide, functions as an intermediate in the formation of more potent and toxic oxidants, such as hypochlorous acid, formed by the action of myeloperoxidase, and the hydroxyl radical, formed by the oxidation of transition metals. These partially reduced metabolites are referred to as reactive oxygen species (ROS) due to their higher reactivity relative to molecular oxygen.

When produced in high concentrations, nitric oxide (NO) also functions as a source of highly toxic oxidants called reactive nitrogen oxide species (RNOS), which are formed via the reaction of NO with superoxide anion or molecular oxygen. These include peroxynitrite ($ONOO^-$), nitroxyl radical, and nitrogen dioxide. The formation of ROS and RNOS is outlined in figure 1.1.

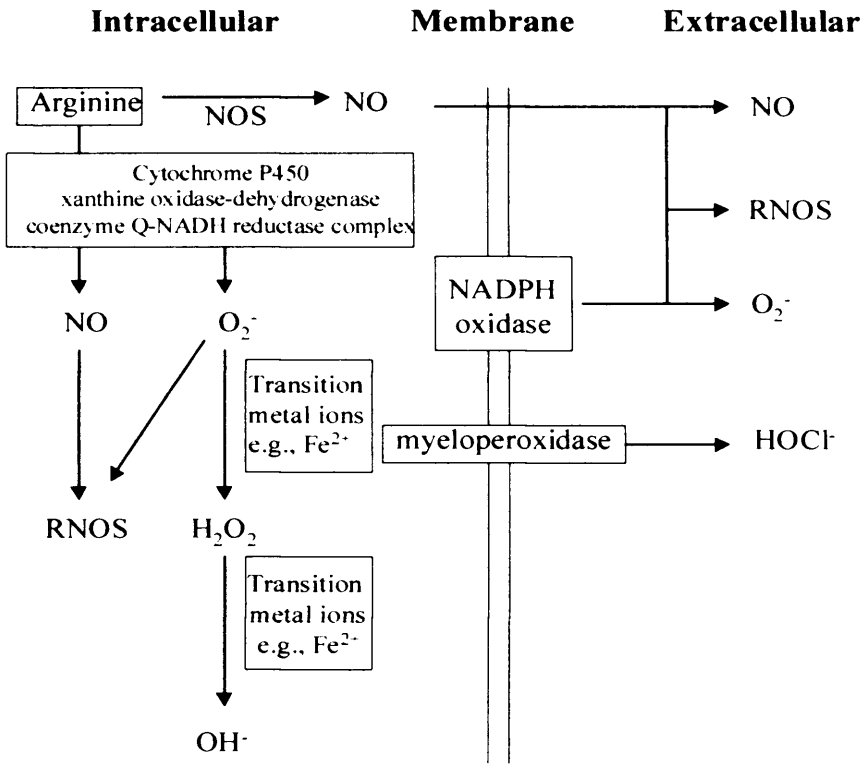


Figure 1.1 Cellular sources of ROS and RNOS (*from Bomzon and Ljubunic, 2001*).

ROS and RNOS have an important positive role as a part of the host immunological defence pathway. These molecules are generated appropriately by macrophages and neutrophils, in order to eliminate microbes and other foreign material. However, production of high levels of ROS and RNOS is potentially damaging to cellular macromolecules including lipids, proteins, and DNA.

Cellular defence mechanisms have evolved to counteract the harmful effects of ROS and RNOS. These antioxidant defence mechanisms can be broadly divided into enzymatic and non-enzymatic systems. Free readical scavenging enzymes include superoxide dismutase (SOD), catalase, and glutathione peroxidase. Non-enzymatic antioxidants include low molecular weight compounds such as reduced glutathione (GSH) and vitamin E. This is further illustrated in figure 1.2.

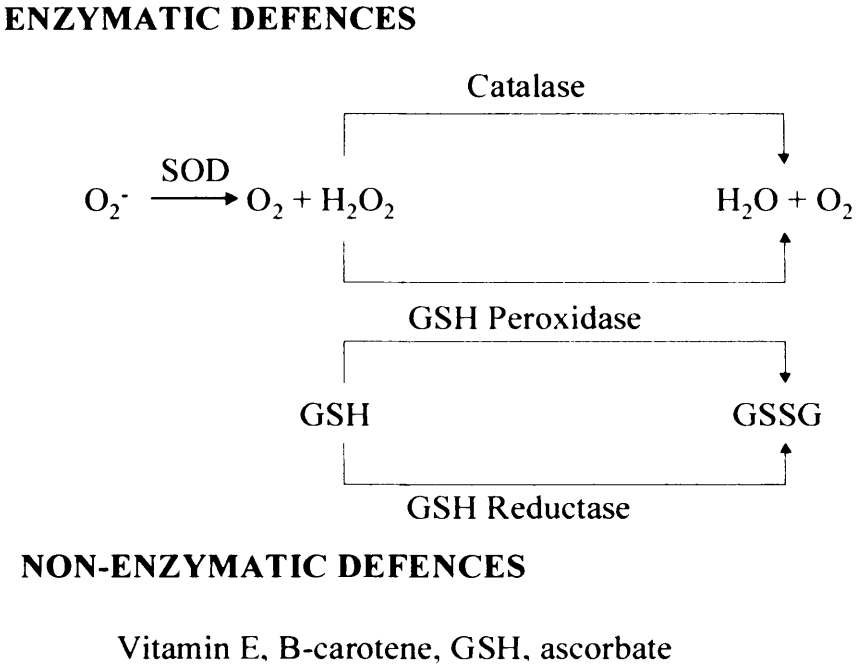


Figure 1.2. The major cellular antioxidant mechanisms.

Therefore the terms oxidative and nitrosative stress may be broadly defined as an imbalance between ROS and/or RNOS production and the antioxidant capacity of the cell to prevent oxidative or nitrosative injury. Overproduction of ROS and RNOS has been implicated in the pathogenesis of numerous chronic inflammatory diseases, atherosclerosis, cancer, diabetes, and the ageing process (*Cross et al. 1987, Halliwell et al. 1992).*

1.1.2 Circulatory Abnormalities in Liver Disease

Advanced liver disease is associated with the development of portal hypertension and a hyperdynamic circulation. Together these changes are responsible for many of the life threatening complications that occur in cirrhosis, including variceal bleeding, the development of ascites and the hepatorenal syndrome, the hepatopulmonary syndrome, and hepatic encephalopathy

1.1.2.i Portal Hypertension

An increase in portal pressure is an early event in liver disease. It results from both an increase in resistance to portal blood flow through the liver and an increase in the volume of portal venous inflow. The latter occurs as a result of the hyperdynamic circulation, which is described in more detail below.

The increase in resistance occurs due to both structural changes, such as nodule formation occuring as a part of the cirrhotic process, and also due to increased resistance at the level of the intrahepatic sinusoidal circulation. Whereas the structural changes are fixed, resistance at the level of the intrahepatic sinusoids is under dynamic control. This was demonstrated by Bhathal and Grossman (*1985*) who showed that infusion of sodium nitroprusside into isolated cirrhotic rat livers, with a constant flow rate, could reduce portal pressure by 15%. They suggested that blood flow was controlled at the sinusoidal level by perisinusoidal stellate cells, which, as a result of liver injury, undergo phenotypic changes from fat storing lipocytes to contractile myofibroblasts. Subsequently it has been shown that vasoconstrictors such as endothelin and thromboxane A2 can cause these hepatic myofibroblasts to contract (*Rockey and Weisiger 1996, Kawada et al. 1992*) and that the vasodilator nitric oxide can lead to their relaxation (*Kawada et al. 1993*). Therefore the relative balance of these vasoactive compounds control sinusoidal blood flow and thus influence portal pressure.

1.1.2.ii The Hyperdynamic Circulation

50 years ago it was first recognised that progressive liver disease is associated with the development of well-defined circulatory abnormalities termed the hyperdynamic circulation (*Kowalski and Abelmann 1953*). This is characterised by a low systemic vascular resistance, high cardiac output, a low mean arterial pressure and an increased circulating blood volume.

The series of events leading from structural changes in the liver, and associated portal hypertension, to the development of these haemodynamic changes remains unclear. Studies of liver disease at various stages throughout its progression from early Childs A to decompensated

Childs C cirrhosis have demonstrated well defined physiological and anatomical stages, prior to the development of the hyperdynamic circulation. An early feature contributing to the development of portal hypertension, in addition to increased hepatic resistance, is portal venous dilatation and an increase in portal venous blood flow (*Benoit and Granger 1986*). Another early feature is subclinical sodium retention resulting in the expansion of all body fluid compartments, including the systemic and central blood volumes (*Bernardi et al. 1993, Wong et al. 1994*). An imbalance in the production of several vasoactive compounds have been described.

Both endogenous vasodilators and vasoconstrictors are overproduced in liver disease. A major problem in interpreting the role of these systems is establishing whether they are primary events or compensatory secondary changes. Another paradox is that regional circulatory differences exist in advanced liver disease. For example, whereas vasodilatation is the main feature in the splanchnic and pulmonary circulation, vasoconstriction tends to predominate in the renal circulation. This may reflect different pharmacological responses to vasoconstrictors that are increased in liver disease, such as the endothelins (*Moore et al. 1992*) and the renin-angiotensin system (*Fernandez-Seara et al. 1989*).

Amongst the vasodilators, there is abundant evidence that overproduction of nitric oxide, either directly or indirectly, is a major contributor to the hyperdynamic circulation. As this compound is a focus of the work presented in this thesis its role will be described in more detail later on in the introduction.

1.1.2.iii Animal Models of The Hyperdynamic Circulation and Portal Hypertension

There are three commonly studied rat models of the hyperdynamic circulation. Two are associated with liver injury, namely secondary biliary cirrhosis, following bile duct ligation, and chronic exposure to carbon tetrachloride. The third model is of partial portal vein ligation, in which the liver itself functions normally. The animal model chosen for the work outlined in this thesis is the bile duct ligated rat.

The bile duct ligated rat has the advantage of being technically simple and of producing cirrhosis within a relatively short period of time. The first documented model of bile duct ligation was in cats in 1637 by Malpigui, who, by tying off the common bile duct and the cystic duct, was able to disprove an earlier hypothesis of the Italian anatomist Fallopius (1523 – 62), that bile was produced in the gallbladder. The earliest study of pathological changes following bile duct ligation in rats was from University College Hospital by Cameron and Oakley (*1932*), who documented cirrhotic change. Subsequently Kountouras *et al* (*1984*) described fully the pathological changes. Within the first 10 days there is an expansion of portal tracts by fibrosis together with bile duct proliferation, the ductular proliferation extending beyond the limits of the portal tracts to invade the liver parenchyme. By 15 days fibrous connective tissue septae bridge portal areas and frequently extend into the lobules. By 28 days the majority of rats develop well defined nodules indicative of cirrhosis, together with the development of ascites and increased spleen weight, consistent with portal hypertension.

1.2 OXIDATIVE STRESS AND THE PATHOGENESIS OF LIVER DISEASE

There are several potential sources of reactive oxygen species across the whole spectrum of clinical and experimental liver disease (*see figure 1.3*). Kupffer cells and newly recruited mononuclear cells and neutrophils release ROS as part of diseases such as alcoholic and viral hepatitis. Pro-inflammatory cytokines such as TNF-α can induce the formation of ROS from hepatocytes. Moreover, ischaemic cell damage can lead to an intracellular oxidant stress, generated by mitochondria and xanthine oxidase, during reoxygenation. Cholestatic liver disease is also associated with oxidant stress, possibly as a result of accumulation of cytotoxic bile acids. Special reference to oxidative stress in the animal model studied in this work, the bile duct ligated rat, will be described in more detail in section 1.5.1.

As reactive oxygen species are highly charged they can react with all biological molecules, including lipids, proteins, nucleic acids and carbohydrates. At a cellular level this leads to a variety of pathological processes with associated liver damage.

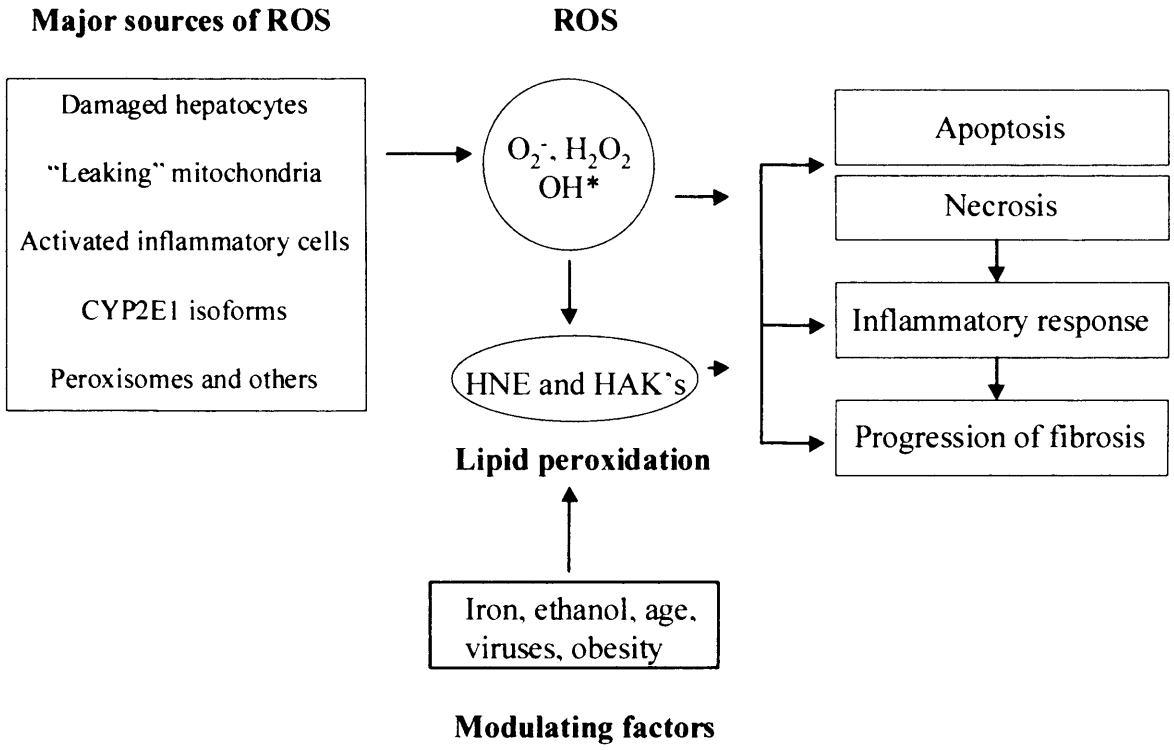


Figure 1.3 Sources of reactive oxygen species (ROS) in liver disease and the pathogenesis of liver disease (*adapted from Parola and Robino 2001*)

1.2.1 Oxidant Stress and Lipid Peroxidation

Lipid peroxidation is initiated by free radical attack on a methylene group (-CH_2-) of polyunsaturated fatty acids. This leads to a variety of end products including 4-hydroxy-2,3-nonenal (HNE) and related 4-hydroxy-2,3-alkenals (HAKs). There is no evidence to suggest that lipid peroxidation *per se* leads to cell death, however products of lipid peroxidation, in particular HAKs, are chemotactic for neutrophils and can induce fibrosis by enhancing collagen gene expression in activated stellate cells (*Casini et al. 1997*).

1.2.2 Oxidative Stress and Apoptosis

Reactive oxygen species have been implicated in the apoptotic cell death of both hepatocytes and endothelial cells in the liver (*Sanchez et al. 1996*). This may be due to changes induced in mitochondrial membrane permeability, a process central both to cell apoptosis and necrosis, or alternatively via an action on caspase enzymes, a family of cysteine proteases that are important for the initiation and progression of apoptosis. It has been demonstrated that caspases can be activated even at low concentrations of hydrogen peroxide (*Herrera et al. 2001).*

1.2.3 Oxidant Stress and Gene Transcription

In addition to inducing direct cell injury and death ROS can affect these processes indirectly by actions on gene expression. One ubiquitous transcription factor of particular importance in immune and inflammatory responses, which is in part under oxidative control, is nuclear factor-κB (NF-κB) (*see figure 1.4*). The activated form of NF-κB is a heterodimer which usually consists of two proteins, a p65 and a p50 subunit. Other subunits may also be a part of activated NF-κB, and differences between these may lead to the activation of different sets of target genes. In unstimulated cells NF-κB is found in the cytoplasm and is bound to an inhibitory factor, IκB, which prevents it from entering the nuclei. When these cells are stimulated, specific kinases phosphorylate IκB, causing its rapid degradation. The release of NF-κB from IκB results in the passage of NF-κB into the nucleus where it binds to specific sequences in the promoter regions of target genes including the inducible form of nitric oxide synthase as well as pro-inflammatory cytokines such as TNF-α and interleukin-1. In addition to ROS many other stimuli activate NF-κB including cytokines and viruses.

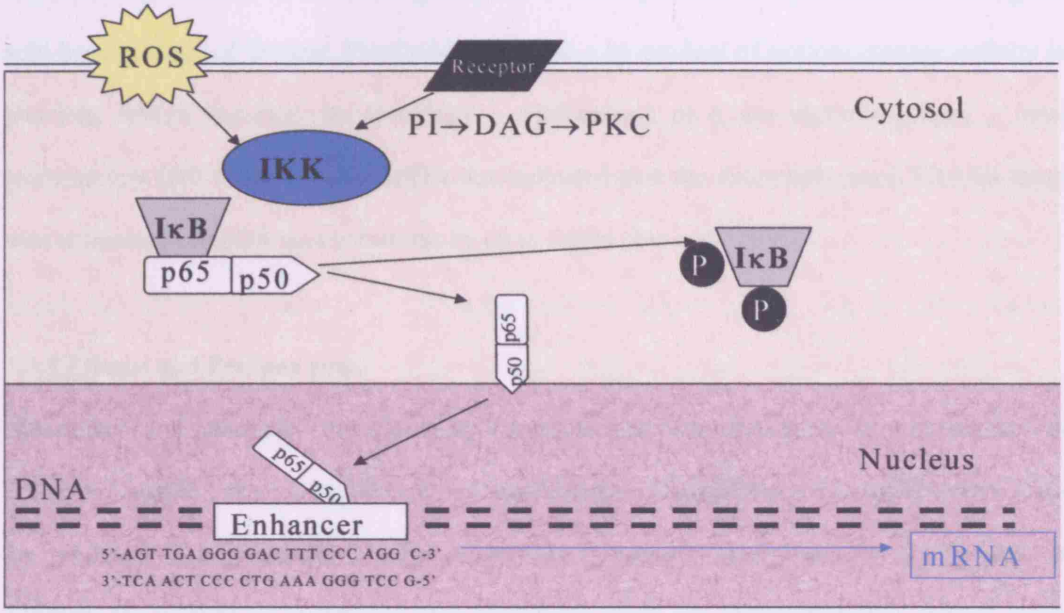


Figure 1.4. Activation of nuclear factor kappa B by reactive oxygen species (*from Holt 2000)*

1.3 MEASUREMENT OF LIPID PEROXIDATION

Free radicals can react with all biological macromolecules. Measurement of lipid peroxidation products is the simplest and most accurate way to quantify ongoing oxidative stress, and can be applied to a wide variety of biological samples including tissue, plasma, and urine. Examples of intermediate or end products of lipid peroxidation that have been applied to clinical and experimental studies include measurement of conjugated dienes, thiobarbituric acid reactive substances (TBARS), exhalation of the alkanes pentane and ethane gases, and cytotoxic aldehydes. The currently accepted gold standard, however, is the measurement of isoprostanes. The methods are briefly reviewed below, with special reference to isoprostanes.

1.3.1 Thiobarbituric acid-reacting substance assay (TBARS)

This involves indirect measurement of malondialdehyde (MDA) concentrations, by measuring absorbance of a pink chromogen at 532 nm, which is formed by the reaction of MDA with TBARS at an acid pH. It is of limited value in biological substances because other aldehydes can also form chromogens with absorbance at 532 nm. Moreover TBARS rarely measure the

free MDA content but rather MDA generated by decomposition of lipid peroxides during the acid-heating stage of the test. Finally MDA is also a by-product of cyclooxygenase activity in platelets, further reducing its specificity. Applications of a gas chromatography / mass spectroscopy (GC/MS) assay for MDA has indicated that the commonly used TBARS assay overestimates true MDA concentrations by up to 6-fold (*Liu et al. 1997*).

1.3.2 Ethane and Pentane gases

Measurement of exhalation of volatile hydrocarbons has been used as an *in vivo* measure of lipid peroxidation. The main issues of concern regarding this method is that hydrocarbon gases are produced by bacteria and are also susceptible to varying rates of *in vivo* metabolism. In addition, the method of separation most commonly used, gas chromatography, cannot easily distinguish pentane from another hydrocarbon, isoprene, due to similar boiling points making interpretation questionable.

1.3.3 Conjugated Dienes

The peroxidation of unsaturated fatty acids is accompanied by the formation of conjugated dienes that absorb ultraviolet light at 230 – 235 nm. Again this methodology suffers from two major drawbacks. Other biological molecules can absorb light at this wavelength and generation of dienes continues to occur *ex vivo* following sampling.

1.3.4 Aldehydes

Following peroxidation of ω-6 and ω-3 polyunsaturated fatty acids, relatively unstable fatty acid hydroperoxides are converted into more stable aldehydes. The major aldehydes formed by peroxidation of ω-6 fatty acids are 4-hydroxynonenal and hexanal, whereas peroxidation of ω-3 fatty acids results in formation of 4-hydroxynonenal and propanol. These aldehydes may be measured by highly specific and sensitive GC/MS.

1.3.5 Isoprostanes

The isoprostanes are a family of compounds produced from polyunsaturated fatty acids via a free radical catalysed mechanism. They are isomers of conventional prostaglandins. Most interest has focused on isoprostanes derived from arachidonic acid, although free radical-derived isomers of other prostaglandins, leukotrienes and thromboxanes have also been reported (*Morrow et al. 1994, Morrow et al. 1996, Harrison and Murphy 1995*). The first demonstration that these compounds were produced *in vivo* came from Morrow *et al.* (*1990*) who reported the discovery of prostaglandin F_2-like compounds, termed the F_2-isoprostanes, which were generated by free radical induced peroxidation of arachidonic acid.

1.3.5.i Structure and Synthesis of Isoprostanes

Isoprostanes differ from conventional prostaglandins in that they are formed, via a cyclooxygenase independent pathway, on intact phospholipids and are released by the action of phospholipases. As is shown in figure 1.5 arachidonyl radicals are formed *in situ* which can then form one of four prostaglandin H_2 isomers. These four prostaglandin H_2 isomers can then be fully reduced to form four prostaglandin $F_{2\alpha}$ regioisomers, or partially reduced to form four prostaglandin E_2 or prostaglandin D_2 regioisomers. As each regioisomer is comprised of eight racemic diastereoisomers, 64 different F_2 and 64 different E_2-isoprostanes can be generated from arachidonic acid (*Cracowski et al. 2001*). There are two current nomenclature systems for isoprostanes in current use (*Taber et al. 1997, Rokach et al. 1997*).

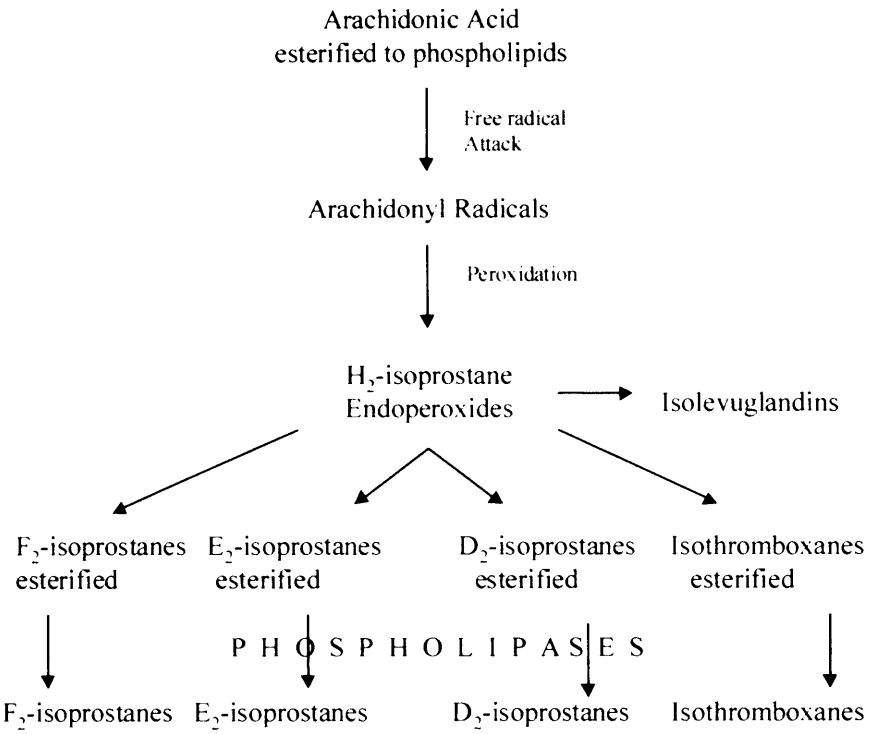


Figure 1.5 The isoprostane pathway (*adapted from Cracowski et al. 2001*)

Among the 64 F_2-isoprostanes the most extensively studied is isoprostaglandin $F_{2\alpha}$ type III (also called 15-F_{2t}-isoP and formerly called 8-iso-$PGF_{2\alpha}$), as this is the most abundant F_2-isoprostane formed *in vivo* under conditions of oxidative stress (*Morrow et al. 1994*). For the rest of the thesis this compound will be referred to by its original name of 8-iso-$PGF_{2\alpha}$ (*see figure 1.6*).

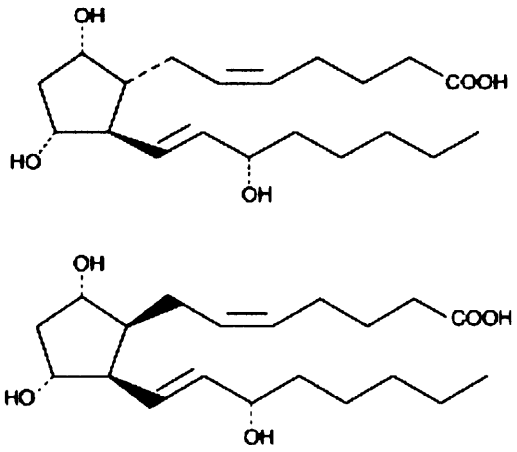


Figure 1.6 The chemical structure of prostaglandin $F_{2\alpha}$ (above) and 8-iso-prostaglandin $F_{2\alpha}$ (below).

Isoprostanes can be measured in any biological material including plasma, urine, bile and tissue samples, and their measurement is now accepted as the most accurate method for the assessment of oxidative stress *in vivo* (*Moore and Roberts 1998*). The gold standard measure has been of 8-iso-$PGF_{2\alpha}$ by gas chromatography mass spectrometry against widely available deuterated standards, though there are also commercially available immunoassay kits. Recently there has been some doubt as to the validity of 8-iso-$PGF_{2\alpha}$ as a pure marker of oxidant injury because *in vitro* cyclooxygenase dependent pathways have been described for formation in platelets and monocytes (*Pratico et al. 1995, Pratico and Fitzgerald 1996*). However, clinical studies have shown that cyclooxygenase inhibitors were unable to decrease the formation of F_2-isoprostanes in healthy subjects (*Wang et al. 1995*). Furthermore, following lipopolysaccharide injection in healthy human volunteers, which increases COX-2 expression, cycloxygenase inhibitors do not effect the production of 8-iso-$PGF_{2\alpha}$ whereas they do decrease production of conventional prostanoids (*McAdam et al. 2000*).

1.3.5.ii Isoprostane Production in Liver Disease

Isoprostane levels have been shown to be elevated in a variety of liver diseases, both in animal models and human studies, consistent with ongoing oxidative injury. See table 1.1.

ANIMAL MODELS	
Acute carbon tetrachloride toxicity - rat	*Morrow et al. 1992*
Alcohol induced liver injury - rat	*Nanjii et al. 1994*
Iron overload - rat	*Dabbagh et al. 1994*
Diquat toxicity in selenium deficiency - rat	*Burk et al. 1995*
Halothane hepatitis - rat	*Awad et al. 1996*
Acute bile duct ligation - rat	*Holt et al. 1999*
Chronic bile duct ligation - rat	*Harry et al. 1999*
HUMAN STUDIES	
Hepatorenal syndrome	*Morrow et al. 1993.*
Biliary obstruction	*Leo et al. 1997*
Alcoholic hepatitis	*Aleynik et al. 1998*
Cirrhotic liver disease	*Pratico et al. 1998*
Alcoholic liver disease	*Hill and Awad 1999*
Hepatitis C	*Jain et al. 2002*

Table 1.1 Liver diseases associated with elevated isoprostane levels.

1.4 ANTIOXIDANT LEVELS IN LIVER DISEASE

Ongoing liver disease and production of reactive oxygen species leads to a depletion in endogenous antioxidants, further contributing to oxidative stress. There have been several studies addressing the levels of endogenous antioxidants in the bile duct ligated rat, the model used in the work in this thesis, and in humans. These are summarised below.

1.4.1 The Bile Duct Ligated Rat

Chronic bile duct ligation is associated with oxidative stress as is shown by an increase in markers of lipid peroxidation as well as a reduction in endogenous antioxidants see table 1.2

MARKER OF OXIDANT STRESS	EFFECT OF BDL	REFERENCE
Plasma and Hepatic Malondialdehyde	↓	A
Hepatic TBARS	↑	C, E
Mitochondrial TBARS	↑	C, D
Plasma Dichlorofluoroscein	↑	B
ANTIOXIDANT	**EFFECT OF BDL**	**REFERENCE**
Vitamin E	↓	A
Hepatic reduced glutathione	↓	A, B, E
Catalase activity	↓	A, B
Glutathione peroxidase and transferase activity	↓	A, B
Superoxide dismutase activity	↓	B
Mitochondrial ubiquinones	↓	C

A) *Singh et al. 1992* B) *Pastor et al. 1997* C) *Krahenbuhl et al. 1995*

D) *Sokol et al. 1991* E) *Panozzo et al. 1995*

Table 1.2 Oxidant stress and antioxidant levels in bile duct ligated rats

The mechanism underlying this increase in oxidative stress is likely to be multifactorial. Following bile duct ligation lipophilic bile acids accumulate in the liver (*Dueland et al. 1991*). At low concentrations the bile acids are incorporated into the mitochondrial membranes with a reduction in phospholipid content (*Krahenbuhl 1993*), changes in membrane fluidity and subsequent impairment of the function of enzyme complexes of the electron transport chain, leading to free radical production. In addition following bile duct ligation there is a significant endotoxaemia and production of proinflammatory cytokines such as TNF-α, which will further contribute to oxidant injury.

1.4.2 Antioxidant Levels in Human Liver Disease

Ongoing oxidative stress with pre-cirrhotic liver diseases such as alcoholic liver disease and hepatitis C has been shown to be associated with depleted antioxidant levels (*Girre et al. 1990, Jain et al. 2002*). Chronic cholestatic liver diseases such as primary biliary cirrhosis are frequently associated with malabsorption of fat soluble vitamins and therefore depleted vitamin E levels, as well as low levels of a variety of other antioxidants. Table 1.3 summarises the evidence for depletion of antioxidants in cirrhotic liver disease.

TISSUE	ANTIOXIDANT	REFERENCE
Liver biopsy	↓ selenium	*Dworkin et al. 1988*
Liver biopsy	↓ superoxide dismutase activity ↓ catalase activity ↔ glutathione peroxidase activity	*Togashi et al. 1990*
Liver biopsy	↓ α-tocopherol	*Bell et al. 1992*
Liver biopsy	↓ α-tocopherol ↓ β-carotene ↓ lycopene	*Leo et al. 1993*
Plasma / erythrocytes	↓ reduced glutathione	*Loguercio et al. 1996*
Plasma / erythrocytes	↓ reduced glutathione ↓ β-carotene ↔ glutathione peroxidase activity ↔ superoxide dismutase activity ↔ α-tocopherol	*Van de Casteele et al. 2002*
Plasma	↓ selenium ↑ glutathione peroxidase activity	*Burk et al. 1998*
Serum	↓ α-tocopherol	*Look et al. 1999*
Erythrocytes	↓ superoxide dismutase activity ↓ glutathione peroxidase activity	*Hadi Yasa et al. 1999*

Table 1.3 Antioxidant levels in human cirrhotic liver disease

1.5 NITRIC OXIDE

In 1987 Palmer *et al.* demonstrated that cultured endothelial cells from pig aortas released NO, with similar characteristics to the previously described endothelium derived relaxing factor (*Furchgott and Zawaadski 1980*), and in a concentration sufficient to induce rapid and transient vasodilatation. In the 17 years since this first description a vast amount of information has been described about the biochemistry, physiology and pathophysiology of nitric oxide, and the enzyme responsible for its synthesis – nitric oxide synthase.

1.5.1 Nitric Oxide Synthase

Three isoforms of nitric oxide synthase (NOS) have been cloned. NOS1 (hereafter referred to as neuronal NOS or nNOS), NOS2 (hereafter inducible NOS or iNOS) and NOS3 (hereafter endothelial or eNOS). All three are dimeric, containing both a reductase and an oxidase domain. The overall reaction catalysed by NOS and the co-factors involved is shown in figure 1.7.

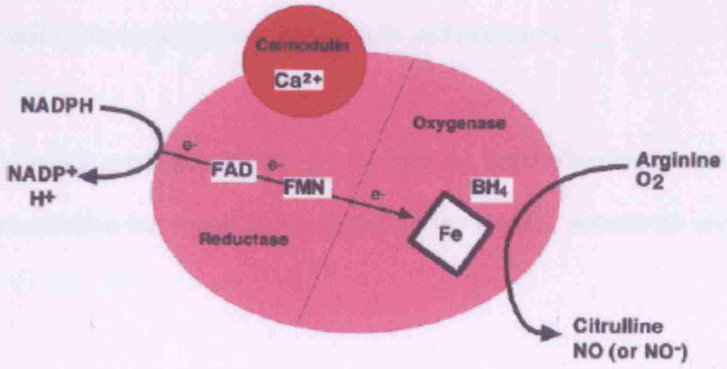


Figure 1.7 Overall reaction catylysed and cofactors of NOS (*from Alderton et al. 2001*).

Electrons (e^-) are donated by NADPH to the reductase domain of the enzyme and proceed via flavine adenine dinucleotide (FAD) and flavine mononucleotide (FMN) redox carriers to the oxygenase domain. There they interact with haem iron and tetrahydrobiopterin (BH_4) at the active site to catalyse the reaction of oxygen with L-arginine, generating citrulline and NO as products. Electron flow through the reductase domain requires the presence of bound Ca^{++} /

calmodulin. It was previously thought that iNOS acted in a calcium independent fashion. It has now been shown, however, that all three isoforms require calmodulin for their enzymic activity (*Bredt and Snyder 1990*), though iNOS requires much lower Ca^{++} concentrations than either eNOS or nNOS.

Inducible nitric oxide synthase (iNOS) is synthesised *de novo* in numerous cell types including macrophages, endothelial cells and vascular smooth muscle cells, following induction by lipopolysaccharide and inflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (*Nathan and Xie 1994*). Once expressed, iNOS synthesises large amounts of NO for extensive periods of time, independent of any haemodynamic or mechanical stimuli.

Endothelial nitric oxide synthase (eNOS), by contrast, releases NO for short periods of time in response to several endogenous and exogenous stimuli, including mechanical factors such as shear stress and receptor-dependent activation of G-proteins by agonists such as acetylcholine and bradykinin. Endothelial NOS expression is not limited to endothelial cells but also is found in many other cell types including mesangial cells and neurones.

Neuronal nitric oxide synthase (nNOS) is activated by depolarisation of nerve endings. This nitronergic transmission has been demonstrated in the central, autonomic and enteric nervous systems.

1.5.2 The Chemistry of Nitric Oxide

The biochemical properties of nitric oxide determine its activity and short half-life ($t_{1/2}$ = 3 – 5 seconds). At room temperature and atmospheric pressure, it is a colourless gas with a solubility in water similar to that of oxygen. It is a free radical by virtue of its unpaired electron, though the NO molecule is not highly reactive *per se*. Its complete lack of reactivity with water gives it a lipophilic character, allowing it to freely diffuse freely through cell membranes. This allows NO to exert its biological effects in a rapid, regional and non-receptor dependent fashion.

Its reactions with other molecules produce a wide variety of complex biochemical interactions and products (*Fukuto 1995*). In the biological milieu the direct chemical interactions of NO are limited to those with transition metals in various oxidation states, with other free radicals and with molecular oxygen. The latter two classes of reactions give rise to more reactive compounds that are capable of triggering a variety of events collectively termed *indirect* effects of NO. This is in contrast to the *direct* effects, in the main reactions with iron-containing prosthetic groups of enzymes such as guanylate cyclase (*see figure 1.8*).

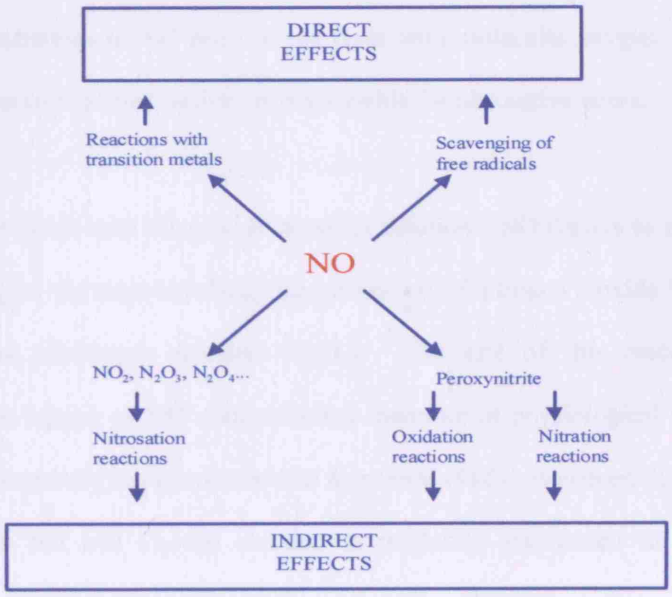


Figure 1.8 Direct and indirect chemistry of NO

One very important principle of nitric oxide chemistry is that at low concentrations of NO (nanomolar), as typically result from activation of eNOS, direct and homeostatic effects tend to prevail, whereas at higher concentrations (micromolar) indirect and deleterious effects tend to prevail. High concentrations are more easily achieved in relation to iNOS expression.

1.5.2.i Direct Effects of Nitric Oxide

In vivo an essential metabolic fate of NO that limits its local concentration is its diffusion into erythrocytes, where it oxidises the ferrous iron of oxyhaemoglobin to yield nitrate anions (NO_3^-)

and methaemoglobin. In the physiological state this mechanism keeps the concentration of NO in the nanomolar range in the non-hydrophobic compartment (i.e outside biological membranes) (*Beckman and Koppenhol 1996*). Under normal physiological conditions the direct effects of NO prevail. The best characterised one is the activation of soluble guanylate cyclase, which in turn mediates a wide variety of physiological responses (*Nathan and Xie 1994*) including smooth muscle relaxation, inhibition of platelet aggregation and inhibition of leucocyte adhesion.

1.5.2.ii Indirect Effects of Nitric Oxide

When high concentrations of NO arise, it can react with molecular oxygen or superoxide and generate highly reactive species, which are responsible for nitrosative stress.

Reaction of Nitric Oxide with Oxygen. In aqueous solutions, NO decays to nitrite (NO_2^-) by a reaction with oxygen, via steps involving the generation of nitrogen dioxide $NO_2^{\bullet}$ and a potent nitrosating species, dinitrogen trioxide (N_2O_3). The rate of this reaction is inversely proportional to the square of NO concentration, therefore at physiological concentrations its importance is at most marginal (*Beckman and Koppenol 1996*). However, due to the lipophilic character of both NO and O_2 the reaction is markedly accelerated in the hydrophobic compartment of biological membranes, where formation of N_2O_3 may increase by a factor of 10^4 in conditions of high NO production (*Miller and Sandoval 1999*).

In human plasma, higher oxides of nitrogen lead to oxidative damage and antioxidant depletion (*Halliwell et al.* 1992). Another important chemical consequence are nitrosation reactions. The major target for nitrosation are thiol groups of proteins and peptides such as glutathione, with the formation of a group of compounds termed the S-nitrosothiols. As they are a focus of the work presented in this thesis they will be discussed in more detail in sections 1.5.4 and 1.9.3.

Reaction of Nitric Oxide with Superoxide. NO rapidly reacts with the superoxide radical (O_2^-) to yield peroxynitrite ($ONOO^-$), a highly reactive oxidant species, at a near diffusion limited

rate. The half life of peroxynitrite is short (~ 1s), but sufficient to allow significant reactions with biomolecules. At physiological levels of O_2^- production, tissue concentrations of superoxide dismutase are sufficient to divert O_2^- away from NO, towards transformation into hydrogen peroxide. Studies in biological systems have shown that peroxynitrite formation is restricted to conditions characterised by abnormally high and approximately equal fluxes of NO and O_2^- (*Miles et al. 1996, Rubbo et al. 1994*).

Peroxynitrite is a major source of oxidant injury, both by initiation of lipid peroxidation (*Hogg and Kalyanaram 1999*) and oxidation of reduced glutathione (*Arteel et al. 1999*). It is capable of irreversibly inhibiting several steps in the mitochondrial electron transport chain (*Brown 1999*) and modifying DNA. Several of its biological effects are related to nitration of tyrosine residues in proteins, particularly enzymes including manganese SOD (*MacMillan-Crow and Thompson 1996*) and prostaglandin I_2 synthase (*Zou et al.1999*). This free radical has also been implicated in the generation of S-nitrosothiols.

1.5.3 Measurement of Nitric Oxide Production

As a result of a greater understanding of the chemistry of NO a variety of methods have evolved that have allowed NO production to be accurately measured in a variety of biological systems.

1.5.3.i Electrochemical Detection

NO is an electrochemically reactive species that can be oxidised at the surface of a metallic carbon or carbon-modified anode.

$$\mathbf{NO \xrightarrow{-e^-} NO^+ \xrightarrow{+OH^-} HONO \xrightarrow{-H^+} NO_2^-}$$

The current generated from the oxidation is directly proportional to the concentration of NO, with a detection limit as low as 10 nmol/L. Electrochemical detection has been used to measure NO release from cultured cells and in addition, a porphyrinic microsensor has been developed

which can measure direct vascular release of NO in the human circulation (*Vallance et al. 1995*).

1.5.3.ii Haemoglobin Oxidation

The reaction of oxyhaemoglobin (HbO_2) with NO to form methaemoglobin (metHb) and nitrate (NO_3^-) has also been used as the basis of an assay to measure NO production.

$$\mathbf{HbFe^{2+}O_2 + NO \longrightarrow HbFe^{3+} + NO_3^-}$$

The rapid reaction of NO and HbO_2 (3.7×10^7 $[mol/L]^{-1}.s^{-1}$) ensures that under most conditions NO will be consumed stoichiometrically.

1.5.3.iii Chemiluminescent Detection

Nitric oxide will react with ozone (O_3) to form nitrogen dioxide (NO_2), a portion of which is in an excited state. As the NO_2 species return to ground state, light is emitted.

$$\mathbf{O_3 + NO \longrightarrow O_2 + NO_2^* \longrightarrow NO_2 + \mathit{hv}}$$

The rapid rate of reaction of NO with ozone ($\sim 10^7$ $[mol/L]^{-1}.s^{-1}$) and the sensitivity of the chemiluminescense detection of the light emitted by NO_2^* (~100 pmol/L) allows real time monitoring of NO production. This method can be employed to measure NO in exhaled breath or production from cell culture systems.

As the majority of NO produced *in vivo* will be rapidly oxidised to NO_2^- and NO_3^-, these metabolites must first be reduced to accurately measure NO production. This can be achieved by using the enzyme nitrate reductase to reduce NO_3^- to NO_2^-, which is further reduced to NO by its reaction with iodide under acidic conditions.

1.5.3.iv Arginine-Citrulline conversion

Nitric oxide synthase catalyses the oxidation of arginine, resulting in the formation of NO and stoichiometric amounts of citrulline. Thus, the net production of NO by a cell preparation or tissue extract can be estimated by the rates of formation of radiolabelled citrulline from radiolabelled arginine, in the presence of saturating concentrations of the NOS co-factors FAD, FMN, NADPH, tetrahydrobiopterin, calcium and calmodulin (in the case of endothelial and neuronal NOS). To initiate the reaction, radiolabelled arginine and cofactors are added to the cell or tissue preparation, the reaction is then terminated by the addition of EDTA at pH 5.5 to bind calcium, resulting in enzyme inactivation. Samples are then eluted on a cation exchange column, which binds arginine but not citrulline, which can then be measured in a liquid scintillation counter.

1.5.3.v The Griess reaction

Nitrite, as a stable product of NO, can be measured in a spectrophotometric assay using the Griess reagents sulfanilamide, HCl and N-(1-napthyl)-ethylenediamine (NED), which together with nitrite produce an azo dye, as illustrated below. To measure total NO accumulation products, any NO_3^- must first be reduced to NO_2^- as above.

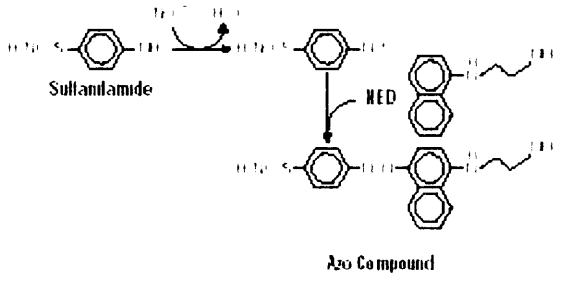


Figure 1.9 The Griess reaction

The reaction can be monitored at A_{548} in a standard spectrophotometer, or it can be adapted for a microplate reader using a 550 nm filter.

1.5.4 Nitric Oxide Transport

The half-life of NO in whole blood has been estimated to be 1.8 msec (*Liu et al. 1998*). In spite of this there is evidence to suggest that NO can produce effects distant to its site of production, consistent with a much longer physiological half-life. For example inhaled NO causes vasodilatation and inhibits platelet aggregation in the peripheral circulation in animal models (*Fox-Robichaud et al. 1998*) and increases renal blood flow in healthy human volunteers (*Wraight and Young 2001*). This is consistent with the hypothesis that carrier molecules exist capable of binding NO and transporting and delivering it within the circulation. The two most likely candidate molecules are haemoglobin and thiol containing peptides or proteins.

1.5.4.i Haemoglobin as a Nitric Oxide Transporter

It has been known for several years that the interaction of NO with oxy-haemoglobin and the subsequent formation of met-haemoglobin and nitrate is a major pathway for NO inactivation and elimination. The rate of NO consumption by red blood cells is, however, much slower than predicted from the rate of NO reaction with free haemoglobin. There are multiple mechanisms, under physiological conditions, which may explain this discrepancy including flow characteristics within blood vessels and the diffusion layer surrounding erythrocytes. Transmembrane and intracellular diffusion are also rate limiting.

Another reason for this apparent discrepancy is that haemoglobin may act as a NO transporter as suggested by Gow and Stamler (*1998*). NO reacts with the haem-Fe (II) moiety of haemoglobin resulting in the formation of iron-nitrosyl-Hb ($HbFe_{II}NO$). Furthermore under condition of high oxgyen tension, as may occur in the alveolar capillaries, allosteric transition of Hb leads to the transfer of NO from the haem group to the cysteine 93 residue of the β chain, forming S-nitroso-haemoglobin (SNO-Hb). Conversely SNO-Hb releases NO when the pO_2 drops, as in peripheral tissues, thus oxygen tension may control NO release via allosteric regulation of haemoglobin, and would therefore, hypothetically, control blood flow to areas of relative hypoxia.

1.5.4.ii S-nitrosothiols

Over the past decade there has been significant interest in a class of compounds termed the S-nitrosothiols, which share many of the physiological properties of NO, but differ in that they have a significantly longer half life. S-nitrosothiols are formed by S-nitrosation of the thiol group or cysteine residues in proteins. They have the general formula RSNO, where R is usually a protein, a peptide or an amino acid. Examples of endogenous S-nitrosothiols include S-nitrosoalbumin (SNO-albumin), S-nitrososglutathione (GSNO) and S-nitrosocysteine (SNO-cysteine).

1.5.4.ii.a Biochemical Properties of S-nitrosothiols

Chemically RSNOs can be considered as the covalent link between NO^+ and a thiolate anion. Depending upon the thiol they differ in terms of their half-lives, for example SNO-albumin has a half –life in buffer solution of hours, whereas that of SNO-cysteine is only minutes.

Nitric oxide release from RSNOs can occur by both heterolytic and homolytic mechanisms. This degradation to produce the corresponding disulphide and NO is enhanced in the presence of free thiols, high oxygen tensions and at a pH > 2. An example of homolytic cleavage is the effect of ultraviolet light and transition metal ions such as copper and mercury.

Ascorbate and copper containing proteins such as caeruloplasmin also decompose RSNOs, which may have important consequences *in vivo.* In addition, enymatic cleavage by γ-glutamyl transpeptidase (*Hogg et al. 1997*), glutathione peroxidase (*Freedman et al. 1995*), and xanthine oxidase (*Trujillo et al. 1998*) have also been described.

Transnitrosation reactions i.e the transfer of NO^+ between thiols and RSNOs have also been suggested to be one mechanism underlying the biological action of RSNOs *in vivo* (*Stamler 1994*).

RSNO + R'SH ↔ RSH + R'SNO

Since the nirosomium ion (NO^{+}) does not react with haem this may in part explain why RSNOs avoid rapid scavenging by haemoglobin.

In vivo, the rate of transnitrosation will be dependent upon the relative concentrations and pKa's of the donor and recipient thiols, and on the relative stabilities of the respective RSNOs (*Singh et al. 1996*) such that intracellularly GSNO formation will be favoured, whereas in plasma the most abundant thiol is albumin, with lower concentrations of glutathione and cysteine. Based upon the calculated kinetics and equilibrium constants of transnitrosation reactions between these thiols, and their known physiological concentrations some authors have suggested that transnitrosation reactions are unimportant (*Meyer et al. 1994*). However, Scharfstein *et al.* (*1994*) showed that the hypotensive effects of infusing S-nitrosoalbumin into rabbits were markedly enhanced and reduced in duration if cysteine was pre-infused, suggesting that transnitrosation reactions are physiologically important, particularly if the resultant RSNO is rapidly metabolised to release NO.

Alb-SNO + RSH ↔ Alb-SH + RSNO → Metabolism

1.5.4.iib Physiological Properties of S-nitrosothiols

S-nitrosothiols show important physiological properties similar to those of NO, including vasodilatation, smooth muscle relaxation, soluble guanylate cyclase activation and inhibition of platelet function.

The importance of transnitrosation reactions in the physiological actions of RSNOs has been shown using synthetic S-nitroso-glutathionyl-Sepharose-4B beads, which at 37°C and pH 7.4

have a half life of 7 days (*Liu et al. 1998*), and are too large to be transported intracellularly. The beads have the same physiological characteristics as low molecular weight RSNOs, albeit in plasma free systems, of vasorelaxation and inhibition of platelet aggregation thereby implicating S-transnitrosation reactions at the cell surface in NO signal transduction pathways.

Although NO release by S-nitrosothiols is clearly important in their mode of action, it may not be the sole mechanism. A study by Ceron *et al.* (*2001*) showed that S-nitrosoglutathione and SNO-N-acetylcysteine cause rat aorta vasodilatation in a manner which was not proportional to NO release. Moreover, they showed that blockade of low conductance potassium channel blockers and K_{ATP} channel blockers partially prevent their action. It is now recognised that S-nitrosation may also involve membrane bound proteins such as ion channels, leading to functional changes in activity. A further example of this is the mechanism by which NO, bound to haemoglobin, is exported from erythrocytes. It has been demonstrated that upon deoxygenation, the NO group is transferred through transnitrosation from the cysteine residue of haemoglobin to a cysteine residue in the cytoplasmic domain of a plasma membrane protein – the anion-exchange protein AE-1 (*Pawloski et al. 2001*). This has lead to suggestions that there may be tissue specific mechanisms for RSNO metabolism.

As previously described the predominant circulating thiol containing protein is albumin. Its role in nitric oxide transport was highlighted in an elegant study by Minamiyama *et al.* (*1996*). Administration of a NO donor NOC-7 to normal rats caused prolonged vasodilatation and hypotension. However, rats which genetically lacked albumin experienced only a transient vasodilatory effect. An interpretation consistent with these data is that immediate relaxation is due to the direct release of NO from the NO donor, and simultaneous with this process is the S-nitrosation of albumin. The prolonged relaxation observed in normal rats was dependent on the subsequent conversion of SNO-albumin to form a species capable of eliciting vessel relaxation, whereas this could not occur in the analbuminaemic rats. This is consistent with the hypothesis that the formation of RSNOs and more specifically SNO-albumin in blood may represent a buffer or storage system being used to transport NO to sites distal to those of its production.

1.6 NITRIC OXIDE AND LIVER DISEASE

Production of nitric oxide in liver disease may have both beneficial and detrimental effects. The balance between these positive and negative outcomes is likely to depend upon a variety of factors, though of importance is the amount of NO produced and the antioxidant capacity of the liver. NO production will be increased as a part of all liver diseases, due to increased cytokine production. A low dose of NO serves to maximise blood perfusion, prevent platelet aggregation and thrombosis. In addition, in acute hepatitis infections and other inflammatory processes NO has important antimicrobial and antiapoptotic properties. However, in cirrhotic liver disease it would appear that there is a sustained upregulation of the inducible NOS isoform with consequent production of relatively high levels of NO. This leads to important haemodynamic consequences as outlined below. Moreover, in the absence of adequate antioxidant defences and an ongoing production of toxic reactive oxygen intermediates formation of peroxynitrite will be favoured. This free radical is capable of altering protein and DNA function, and contributes to cellular injury.

This dual effect of NO was illustrated in a recent study looking at progression of liver fibrosis, following bile duct ligation (Mayoral *et al. 1999*). In this study both augmentation and inhibition of NO production, with arginine and L-NAME respectively, lead to increased fibrosis and liver damage. In another study Diez Fernandez *et al.* (*1998*) showed that NOS inhibition with aminoguanidine markedly reduced liver injury following administration of thioacetamide.

The overproduction of NO in cirrhosis has important cardiovascular effects and is almost certainly of importance in the pathogenesis of the hyperdynamic circulation, as will be discussed below.

1.6.1 Nitric Oxide and the Hyperdynamic Circulation

In 1991 Vallance and Moncada proposed that increased synthesis and release of NO, secondary to upregulation of iNOS, in turn secondary to endotoxaemia and increased cytokine production, was responsible for vasodilatation and the hyperdynamic circulation in chronic liver disease. Their hypothesis was based upon the following evidence: 1) NOS is induced in the endothelium and smooth muscle when vascular tissue is exposed to endotoxin or cytokines *in vitro* (*Busse and Mulsch 1990*). 2) Infusion of endotoxin into humans leads to the gradual appearance of peripheral vasodilatation (*Suffredini et al 1989, Fong et al. 1990*). 3) High circulating levels of endotoxin are found in cirrhotic patients with or without clinical evidence of infection (*Lumsden et al. 1988*).

Over the subsequent decade there have been three principle lines of evidence supporting overproduction of nitric oxide in patients and experimental animal studies. First, products of nitric oxide metabolism, such as nitrite and nitrate, are elevated in liver disease, second vascular tissue shows an NO dependent hyporesponsiveness to vasoconstrictors and third NOS inhibitors are capable of partially reversing the hyperdynamic circulation.

Identification of the isoform responsible for this increased production has been more contentious. In agreement with the Vallance and Moncada hypothesis, several studies have shown increased iNOS activity in liver disease, although others have suggested that upregulation of the eNOS isoform is responsible for increases in NO production. This has lead to suggestions that NO overproduction may actually be partly an effect rather than directly a cause of the hyperdynamic circulation, with the stimulus to eNOS upregulation being increased shear stress, secondary to architectural and vascular distortion occuring as a part of the natural history of cirrhosis itself (*see figure 1.9*).

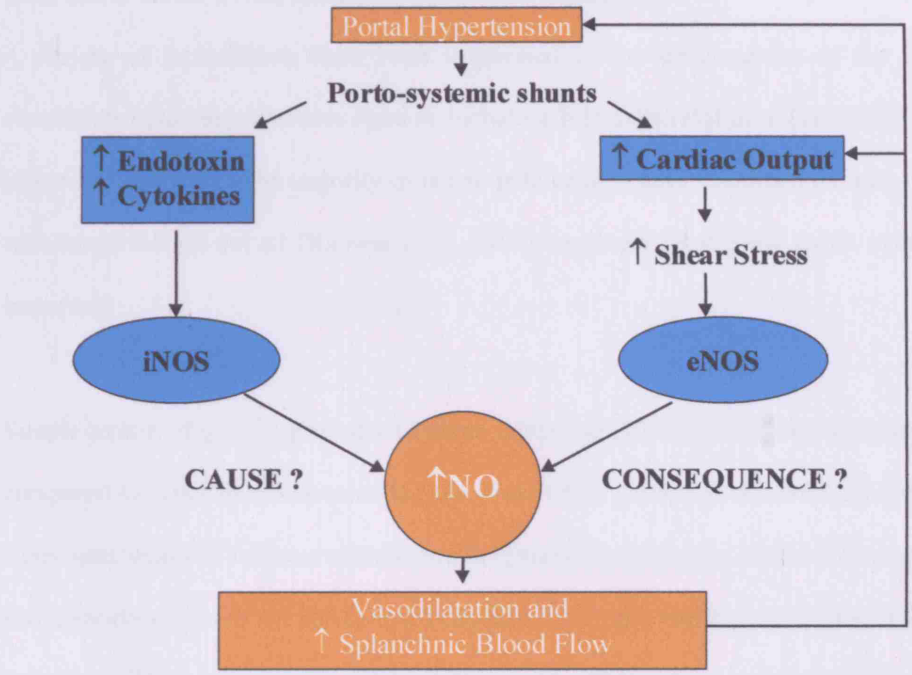


Figure 1.10 Nitric oxide and the hyperdynamic circulation

One explanation for these apparent disparities is the recently discovered link between cytokines, endotoxaemia and increased eNOS activity. Tetrahydrobiopterin (BH_4) is an essential and rate-limiting cofactor in nitric oxide production. Upregulation of BH_4 directly increases the activity of eNOS in the absence of a detectable increase in eNOS levels (*Wever et al. 1997*). Moreover, expression of the rate-determining enzyme in the synthesis of BH_4, GTP cyclohydrolase 1, is under the control of proinflammatory cytokines (*Rosenkranz-Weiss et al. 1994, Walter et al. 1998*). In the context of liver disease, Wiest *et al.* (*1999*) have demonstrated that bacterial translocation in cirrhotic rats is associated with increased NO related vascular hyporeactivity together with elevated levels of BH_4 and increased eNOS activity.

There have been several publications examining the role of nitric oxide in the development of the hyperdynamic circulation. The following discussion will be limited to studies of rats with secondary biliary cirrhosis and humans with cirrhotic liver disease.

1.6.2 Nitric Oxide Production in the Bile Duct Ligated Rat

A variety of vasodilators have been implicated in the development of the hyperdynamic circulation following bile duct ligation including bile salts (*Pak and Lee 1993*) and glucagon (*Ohara et al. 1993*). The majority of recent publications have examined the role of nitric oxide with most, though not all (Kanwar *et al.* 1996) concluding that nitric oxide overproduction is important.

Simple assays of plasma products of nitric oxide metabolism have shown higher levels when compared to sham operated animals (*Liu et al. 1999, Criado et al. 1997, Heller et al. 2000*). Consistent with the Vallance and Moncada hypothesis endotoxin levels (*Clements et al. 1998*) and a variety of proinflammatory cytokines have also been shown to be higher in BDL animals, including TNF-α (*Liu et al. 1999, Heller et al. 2000*), IL-6 and IL-1β (*Liu et al, 2001*). Moreover Schmandra *et al.* (*2001*) showed that incubating serum from cirrhotic bile duct ligated rats with aortic rings obtained from normal rats lead to a hyporesponsiveness to the vasoconstrictor phenylepherine in the normal rat vessels, which could be inhibited by a nitirc oxide synthase inhibitor. This would suggest a circulating factor, perhaps endotoxin or cytokines, leading to upregulation of nitric oxide production.

A nitric oxide dependent hyporesponsiveness to vasoconstrictors has been well documented in aortic rings from bile duct ligated animals (*Ortiz et al. 1996, Kimpel et al. 1998*). Splanchnic vasodilatation is a prominent feature of the hyperdynamic circulation, and consistent with a role for NO, Pateron *et al.* (*1999*) found that in BDL cirrhotic rats the aorta and superior mesenteric artery exhibited significant nitric oxide dependent hyporeactivity to phenylepherine, whereas the carotid artery did not. Further evidence for a role for NO overproduction comes from Criado *et al.* (*1997*) and Pilette *et al.* (*1996*) who demonstrated that nitric oxide synthase inhibitors significantly reversed the circulatory changes associated with cirrhosis in this model.

Measurement of the changes in expression and activity of the different isoforms of NOS following experimental liver injury has lead to many conflicting results which appear partly related to the nature of the liver injury and also to its duration. Rockey and Chung (*1997*) found that iNOS could not be detected in any cell types in the liver following chronic carbon tetrachloride induced cirrhosis (though it was detected in Kupffer cells following a single dose) whereas it was found in all non-parenchymal cell types after bile duct ligation. As both iNOS and eNOS are subject to significant post-translational modification, protein and mRNA measurements *per se* are of little value by comparison to measurements of activity by way of the arginine – citrulline conversion assay. A recent study by Wei *et al.* (*2002*) in rats with secondary biliary cirrhosis showed that eNOS activity in the liver was reduced by approximately one half, although levels of eNOS protein or mRNA were actually increased. In contrast, activity of iNOS was markedly increased by comparison to sham operated animals. One explanation for this decrease in eNOS activity may relate to increased binding of the protein caveolin-1, which reduces eNOS activity. Recent work by Shah *et al.* (*1999*) using a different experimental model of cirrhosis has shown that reduced eNOS activity in cirrhotic livers was associated with increased caveolin binding.

Nitric oxide synthase expression and activity has also been examined in a variety of other tissues in animals with secondary biliary cirrhosis, as outlined in table 1.4. Upregulation of NOS activity in these tissues has been proposed to underly the development of cirrhotic cardiomyopathy, hepatopulmonary syndrome and the maintenance of renal blood flow in cirrhosis.

TISSUE	RESULT	REFERENCE
Aorta	No change in eNOS mRNA No detection of iNOS mRNA	*Sogni et al. 1997*
	↑ eNOS mRNA & protein No detection of iNOS mRNA or protein	*Liu et al. 1999*
	↑ total & iNOS activity ↑ iNOS protein ↑ eNOS protein	*Tazi et al. 2002*
Leucocytes	↑ iNOS expression in peripheral lymphocytes	*Criado et al. 1997*
	↑ iNOS activity in peritoneal macrophages	*Heller et al. 2000*
Kidney	↑ glomerular NO production ↑ glomerular iNOS mRNA & protein	*Criado et al. 1997*
	↑ glomerular iNOS mRNA & protein ↑ mesangial cell iNOS immunostaining	*Porst et al. 2001*
Heart	↑ iNOS mRNA in cardiac myocytes	*Liu et al. 2000*
Lungs	↑ exhaled NO levels ↑ total pulmonary NOS activity ↑ iNOS in pulmonary intravascular macrophages	*Nunes et al. 2001*

Table 1.4 Extrahepatic nitric oxide production in BDL rats

1.6.3 Nitric Oxide Production in Human Liver Disease

The role of nitric oxide in the development of the hyperdynamic circulation has also been extensively examined in patients with cirrhosis. Serum and urinary nitrite and nitrate levels are elevated proportionally to the severity of the liver disease and the degree of haemodynamic compromise (*Sanchez-Rodriguez et al. 1998, Hori et al. 1996*). However, Heller *et al.* (*1999*)

have shown that caution must be applied to interpretation of serum levels as they are influenced by both diet and renal function. This was further confirmed in a study suggesting that serum products of nitric oxide were proportional to renal clearance rather than overproduction in cirrhotic patients with ascites (*Campillo et al. 1996*). Nevertheless studies of exhaled NO, which are not influenced by either of the above factors, have shown levels to be proportional to the cardiac index, arteriolar-alveolar oxygen gradient and Childs-Pugh score (*Rolla et al. 1997, Soderman et al. 1997, Matsumoto et al. 1995*). Moreover, these levels revert to normal following liver transplantation (*Rolla et al. 1998*).

In the earliest study of the vascular effects of infusion of the nitric oxide synthase inhibitor L-NMMA (*Calver et al. 1994*), no significant difference was demonstrated between controls and patients with cirrhosis, however, only Childs A and B patients were studied. Subsequently, a role for NO overproduction has been strengthened by similar studies in more advanced patients which have shown significant reversal of the abnormalities in haemodynamic parameters, forearm blood flow and renal blood flow (*Campillo et al. 1995, Forrest et al 1995, LaVilla et al. 2001*) in this group.

As with animal models there continues to be debate as to whether the predominant source of NO production is from the eNOS or the iNOS isoform, and the site of overproduction. In accordance with the initial Vallance and Moncada hypothesis elevated levels of proinflammatory cytokines and endotoxin have been found in patients with decompensated cirrhosis (*Genesca et al. 1999, Hori et al. 1996*). Overproduction of NO in the splanchnic circulation has been demonstrated by EPR spectroscopic measurement of nitrosyl-haemoglobin in patients undergoing transjugular intrahepatic porto-systemic shunting for portal hypertension. Highest levels were found in the hepatic vein followed by the portal vein and then the systemic circulation (*Battista et al 1997*). Examination of tissue taken at the time of liver transplantation by one group showed increased iNOS activity and expression in cirrhotic livers but no difference in eNOS activity (*McNaughton et al. 2002*), whereas another demonstrated increased immunohistochemical staining for both eNOS and caveolin-1 (*Yokomori et al. 2002*). In

addition, increased total NOS activity has been found in hepatic arteries and portal veins from cirrhotic patients which in turn was proportional to the degree of haemodynamic disturbance (*Albornoz et al. 2001*).

Several studies have examined leucocyte nitric oxide production, and nitric oxide synthase activity and expression in cirrhosis. Both lymphocytes and neutrophils nave been shown to express more iNOS than controls and to produce higher amounts of nitric oxide, again proportional to the severity of the hyperdynamic circulation (*Laffi et al. 1995, Sanchez-Rodriguez et al. 1998, Galley et al. 1998*). In turn this increased expression of iNOS is associated with increased mRNA levels for both TNF-α and TNF receptor in lymphocytes (*Hanck et al. 2000*). This increased NO production by leucocytes has been suggested to be important in antimicrobial defence, in particular in peritoneal macrophages (*Jimenez et al. 1999*), but also may be an important contributor to the overall increase in NO production seen in cirrhotic patients.

1.7 ANTIOXIDANT THERAPY IN LIVER DISEASE

Antioxidant treatment has been studied in a variety of animal models of liver disease and clinical settings. The outcomes have been measurement of markers of oxidant injury, in particular of lipid peroxidation, histological improvement in disease progression, changes in biochemical parameters or clinical end points such as death and episodes of decompensation. Again, the presented evidence will be limited to rats undergoing bile duct ligation and patients with liver disease.

1.7.1 Antioxidant Therapy in Bile Duct Ligated Rats

Treatment with a variety of antioxidants has been shown to lead to a reduction in lipid peroxidation and replenishment of antioxidants in rats with secondary biliary cirrhosis. For example *N*-acetylcysteine increases levels of hepatic reduced glutathione and reduces both lipid peroxidation and production of reactive oxygen intermediates (*Pastor et al. 1997*). In one study

it also lead to a reduction in portal pressure (*Bravo et al. 1997*). The glutathione precursor S-adenosyl methionine has been demonstrated to improve ultrastructural changes by electron microscopy (*Muriel et al. 1994*), liver microsomal function (*Pastor et al. 1996*) as well as biochemical and histological parameters (*Bravo et al. 1997*). Although vitamin E supplementation has not been demonstrated to be effective, rats fed on a vitamin E depleted diet had enhanced levels of products of lipid peroxidation and worse liver function (*Sokol et al. 1991*).

1.7.2 Antioxidant Therapy in Human Liver Disease

Antioxidants, both natural and synthetic, have been used as therapeutic agents in a number of human liver diseases. A large proportion of the studies have looked at laboratory based end points of oxidant injury rather than clinical progression of the disease itself. For example vitamin E supplementation has been shown to suppress urinary isoprostane excretion in patients with cirrhosis (*Ferro et al. 1999*) and a grapeseed derived polyphenolic antioxidant has been shown to reduce collagen expression in human myofibroblasts (*Godichaud et al. 2000*).

The best treatment candidates for antioxidant therapy are likely to be liver diseases where steatosis and lipid peroxidation are a major feature, particularly when therapy is started before the development of significant fibrosis. There is increasing interest in the use of antioxidants in the treatment of non-alcoholic fatty liver disease. In this context, small pilot studies using N-acetylcysteine, betaine and vitamin E treatment have all shown significant improvement in liver function tests (*Gulbahar et al. 2000, Abdelmalek et al. 2001, Lavine 2000*). In the study using betaine, which increases s-adenosylmethionine levels, in addition to improvement in laboratory values there was also an improvement in steatosis, necroinflammatory activity and fibrosis on serial liver biopsies. However, this was a small study involving only 10 patients. In addition, there have been two small controlled studies of the use of antioxidants in the treatment of hepatitis C. In one vitamin E alone significantly improved transaminase levels, but in the other using a combination of vitamin E and N-acetylcysteine there was no difference biochemically or virologically versus controls (*von Herbay et al. 1997, Ideo et al. 1999*).

In advanced cirrhotic liver disease there have been 6 clinical trials published on the effectiveness of antioxidant therapy. Of these 4 looked at silymarin, 1 at vitamin E and 1 at s-adenosylmethionine. None of these studies showed any clinical benefit. A summary of these results is given in table 1.5.

ANTIOXIDANT	TRIAL DESIGN	PATIENTS	OUTCOMES	REFERENCE
Silymarin	Double-blind placebo controlled – 2 years	170 with all forms of cirrhosis	No overall benefit Subanalysis – benefit in patients with alcoholic cirrhosis and Childs A	*Ferenci et al. 1989*
Vitamin E	Double- blind placebo controlled – 1 year	67 with alcoholic cirrhosis	No difference in LFTs / mortality or hospitilisation rates	*De la Maza et al. 1995*
Silymarin	Double-blind placebo controlled – 2 years	125 with alcoholic cirrhosis	No benefit on survival	*Pares et al. 1998*
S-adenosylmethionine	Double-blind placebo controlled – 2 years	123 patients with alcoholic cirrhosis	No overall benefit Subanalysis – Childs A/B Improved survival and delay to transplantation	*Mato et al. 1999*
Silymarin	No placebo – 1 year	27 patients with primary biliary cirrhosis	No benefit on LFTs or Mayo score	*Angulo et al. 2000*
Silymarin	Placebo controlled – 6 months	49 with alcoholic cirrhosis	↑ in glutathione levels ↓ in lipid peroxidation No changein LFTs	*Lucena et al. 2002*

Table 1.5 Clinical trials of antioxidants in cirrhosis

1.7.3 Lipoic Acid

The antioxidant α-lipoic acid and its reduced form dihydrolipoic acid theoretically would be an ideal treatment for liver disease, in view of its chemistry and its pharmacokinetics. The naturally occuring R-enantiomer of lipoic acid is an essential cofactor in α-ketoacid dehydrogenase complexes and the glycine cleavage system. The most abundant plant sources are spinach, broccoli, and tomatoes respectively whereas in animal tissues concentrations are highest in kidney followed by heart and liver.

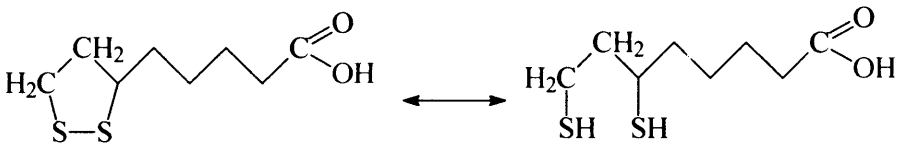


Figure 1.11 Lipoic acid and dihydrolipoic acid

Lipoic acid is a low molecular weight thiol with several important antioxidant functions. It is readily absorbed and taken up into cells where it is interconverted with its reduced form, dihydrolipoic acid (*see figure 1.11*). These two forms of lipoic acid are capable of scavenging hydroxyl, hypochlorous and peroxyntirite radicals directly in the fluid phase, as well as being able to regenerate levels of the antioxidant vitamins C and E, and cellular glutathione. In addition it increases the activity of glutathione reductase, as well as other glutathione metabolic enzymes (*Arivazhagan et al. 2001*), maintaining the overall redox state of cells. *In vitro* studies on the effects of orally supplemented lipoic acid in healthy human volunteers have shown a reduction in oxidative stress as measured by F_2-isoprostanes, plasma protein carbonyls and LDL oxidizability (*Marangon et al. 1999*).

One of the most interesting properties of lipoic acid is its ability to inhibit activation of the transcription factor NFκB (*Suzuki et al. 1992*). Since the binding site for NFκB exists in the promoter region of iNOS, the role of α-lipoic acid in the upregulation of iNOS has been

investigated. Upregulation of iNOS in hepatocytes treated with LPS and cytokines can be effectively blocked by pretreatment with lipoic acid (*Liang and Akaike 2000*). The mechanism behind this is not clear, as other thiol containing antioxidants such as glutathione and N-acetyl cysteine do not have the same effect. This effect has also been demonstrated in human studies. In diabetic patients treated with lipoic acid 600mg per day for 3 days there was a 38% reduction in NFκB binding activity in ex vivo isolated peripheral blood mononuclear cells (*Hofmann et al. 1999*).

Studies on the distribution of radioactivity in rat tissues after intraperitoneal and oral administration of [^{14}C]- or [^{35}C]-lipoic acid have shown that it is readily absorbed in the gut and that the liver has a high capacity for uptake and accumulation (*Bustamante et al. 1998*). So far, studies in animal models have shown a benefit in CCl_4 and cadmium poisoning, both of which are mediated via production of reactive oxygen intermediates. In clinical practice its use has been advocated in liver disease since the 1950's with early suggestions that it was effective in the treatment of hepatic coma, hepatitis, and acute alcohol intoxication. There has to date only been one double blind clinical trial of its use in the treatment of alcoholic liver disease with no benefit being found above placebo treatment in the prevention of histological disease progression (*Marshall et al. 1982*). In addition it has been extensively used in the management of hepatotoxicity secondary to mushroom poisoning, in particular *Amanita phalloides*, however, a meta-analysis of over 200 cases did not show any difference in mortality. (*Floersheim 1987*).

1.8 OXIDATIVE STRESS AND VASOACTIVE COMPOUNDS

A role for oxidative stress in the development of the hyperdynamic circulation was first proposed in work carried out in our laboratory, though not presented in this thesis (*Fernando et al. 1998*). In this study rats underwent partial portal vein ligation (PPVL) and were injected with N-acetylcysteine twice daily for 14 days. Untreated rats developed presinusoidal portal hypertension with a hyperdynamic circulation and oxidative injury shown by enhanced urinary excretion of F_2-isoprostanes. Treated rats did not develop a hyperdynamic circulation, had lower portal pressure and significantly lower isoprostane excretion.

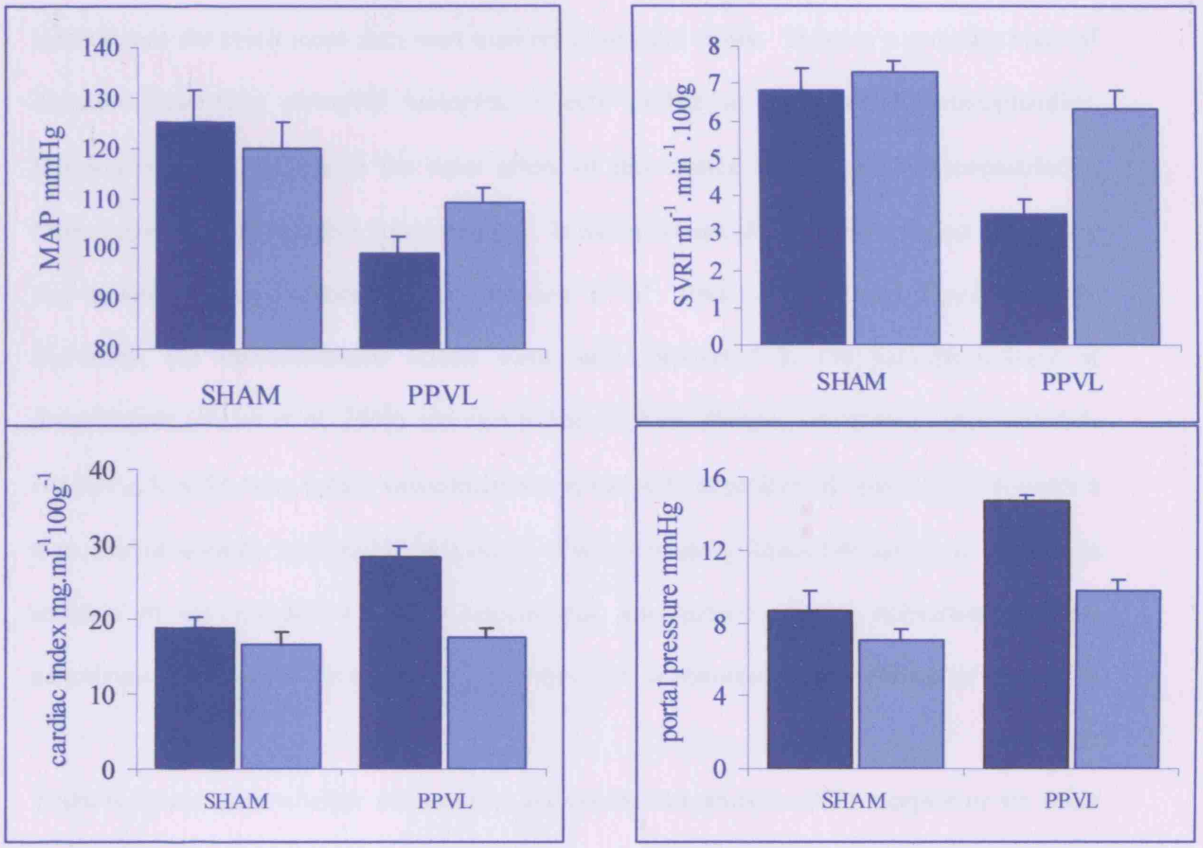


Figure 1.12 The effect of N-acetylcysteine on PPVL rats (*adapted from Fernando et al. 1998*)

As an extension of these findings the work presented in this thesis looks at the interaction between oxidative and nitrosative stress and vasoactive compounds in advanced liver disease. Two classes of vasoconstrictor , one putative – the isoprostanes, and one established – the endothelins, will be discussed; specifically looking at their role in control of the intrahepatic circulation and portal hypertension. In addition the effect of oxidative injury on production of nitric oxide and the role of the S-nitrosothiols in the context of liver disease will also be examined.

1.8.1 The Isoprostanes

Isoprostanes are much more than inert markers of oxidant stress. There is a growing body of literature describing powerful biological effects similar to conventional prostaglandins. Infusion of 8-iso-$PGF_{2\alpha}$ into the renal artery of rats causes intense renal vasoconstriction (*Takahashi et al 1992*) at low concentrations. It has also been shown to be a potent pulmonary and coronary artery vasoconstrictor (*Janssen et al. 1993, Kromer and Tippins, 1996*). Moreover, the vasoconstrictor effects have been implicated in the pathophysiology of preeclampsia (*Walsh et al. 2000*) and also ischaemic heart disease - it has been shown that 8-iso-$PGF_{2\alpha}$ is a far more potent vasoconstrictor in rats with experimental hypercholesterolaemia than control animals, presumably as a result of receptor upregulation (*Wilson et al. 1999*). In addition to the vasoactive effects isoprostanes also induce platelet activation, increase adhesiveness and reduce the antiplatelet activity of NO at nanomolar levels *(Minuz et al. 1998*).

There is debate as to whether isoprostanes act via the thromboxane (TP) receptor or via other prostaglandin (EP and FP) receptors or even via a novel and as yet undescribed isoprostane receptor. TP receptor antagonists completely block 8-iso-$PGF_{2\alpha}$ induced vasoconstriction in a variety of vascular beds including rat preglomerular arteries (*Takahashi et al. 1992*) and cerebral arterioles (*Hoffman et al. 1997*). However, 8-iso-$PGF_{2\alpha}$ displaces the binding of TP receptor homoligands with much lower potency (2 – 3 orders of magnitude) than the homoligands , but stimulates inositol triphosphate production with a much higher potency than TP agonists themselves (*Fukunaga et al. 1993a, Fukunaga et al. 1993b*). Other competitive binding studies have suggested two distinct binding sites, one a high affinity site, likely to be an isoprostane specific receptor, and the other a low affinity site, likely to be the thromboxane receptor (*Fukunaga et al. 1997*). Recently this idea has been challenged with the development of a TP receptor knock out mouse (*Audoly et al. 2000*). In the knock out mouse 8-iso-$PGF_{2\alpha}$ infusion had no effect on blood pressure or platelet aggregation in contrast to the wild type mice. Furthermore, mice engineered to overexpress the TPβ isoform in the vasculature had a significantly greater pressor response to infusion than the wild type mice.

1.8.2 The Endothelins

The endothelins are a group of three physiologically active 21 amino acid peptides, which predominantly act as vasoconstrictors. Their pathway is outlined in figure 1.13. In brief, a large precursor protein, preproendothelin, is transcribed in response to a variety of stimuli which is then cleaved by furin-like endopeptidases to form 41 amino acid biologically inactive intermediates, termed big endothelins. In turn, these are cleaved by endothelin converting enzymes to form the biologically active final products, classified as endothelin 1, 2, and 3.

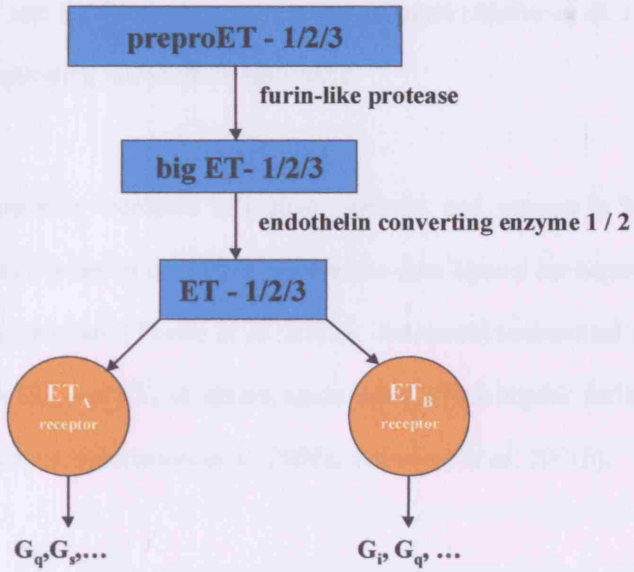


Figure 1.1.3 The endothelin pathway

Two endothelin (ET_A and ET_B) receptors have been identified, both of which activate a set of overlapping G proteins. The ET_A receptor has subnanomolar affinities for ET-1 and ET-2, and an affinity greater than two orders of magnitude lower for ET-3. ET_B has equal subnanomolar affinities for all three peptides.

In vivo the predominant source of ET-1 is endothelial cells, ET-2 is expressed by intestinal epithelial cells and ET-3 by a variety of cells including neurones and renal tubular cells. The

concentration of ET-1 in plasma is ≈ 1 pM, and of ET-2 and ET-3 even lower, which is two orders of magnitude below the pharmacological threshold. Therefore, under normal physiological conditions, endothelins are not circulating hormones, rather they act as autocrine and paracrine factors at multiple sites in the body.

1.8.2.i Endothelins and Liver Disease

Plasma endothelin levels are elevated in animal models of liver injury including the bile duct ligated rat (*Kojima et al. 2000*) and in patients with cirrhosis. Circulating levels of ET-1 correlate with the severity of liver disease (*Tsai et al. 1995*) and in addition hepatic venous levels of both ET-1 and ET-3 correlate with portal pressure (*Moller et al. 1995*), suggesting an important role in controlling intrahepatic resistance.

Endothelin-1 expression is increased in human cirrhosis, and appears to be particularly so in hepatic stellate cells (*Pinzani et al. 1996*). In the bile duct ligated rat hepatic endothelin levels correlate with portal pressure (*Tieche et al. 2001*). Immunohistochemical studies have shown upregulation of both ET_A and ET_B receptors, again especially in hepatic stellate cells in both rats and humans with cirrhosis (*Yokomori et al. 2001a, Yokomori et al. 2001b*).

There is evidence that endothelins contribute to the elevated intrahepatic resistance seen in cirrhosis, most likely due to vasoconstriction secondary to activated stellate cell contraction. Stellate cells isolated from cirrhotic livers, and displaying a myofibroblast phenotype contract in a dose dependent fashion to exogenous endothelin (*Rockey and Weisiger, 1996*). The relative contribution of the two endothelin receptors is unclear. However, three studies have shown that infusion of combined endothelin receptor antagonists significantly reduce the portal pressure in rats with secondary biliary cirrhosis (*Reichen et al. 1998, Kojima et al. 2000, Kojima et al. 2001*).

There is evidence to suggest that bacterial infections can precipitate variceal bleeding in cirrhosis (*Bernard et al. 1995*). It has been suggested that this may be due to an increase in production of endothelins (*Goulis et al. 1999*) causing a rise in portal pressure. In animal models following exposure to endotoxin, endothelin mRNA in liver sinusoidal endothelial cells and plasma endothelin levels are increased by up to ten-fold (*Eakes et al. 1997*). Moreover, infusion of lipopolysaccharide into rats causes an increase in resistance to blood flow in the portal vein, an effect that can be abrogated by administration of the combined ET_A and ET_B receptor antagonist bosentan (*Pannen et al. 1996*).

One theory behind the pathogenesis of alcoholic liver disease is that ethanol causes hypoxic injury to the liver as a result of impaired microcirculatory blood flow. It has been postulated that this may be due to local endothelin release and subsequent vasoconstriction at the level of the hepatic sinusoids. Ethanol at a dose of 0 – 100 mM causes a dose dependent release of endothelin 1 and 2 from human umbilical vein endothelial cells (*Tsuji et al. 1992*). Moreover, infusion of ethanol into the portal vein of normal rats causes an increase in portal pressure, an effect which can be partially inhibited by co-infusion of endothelin-1 antiserum (*Oshita et al. 1993*). This occurs at the same levels of ethanol that have been documented in heavy alcohol consuming humans (*Hamlyn et al. 1975*). Scanning electron microscopy and laser Doppler flow probes have shown that this rise in portal pressure is due to vasoconstriction occuring at the level of sinusoidal blood flow. Moreover, this response can be significantly reduced by endothelin receptor blockade (*Takashimizu et al. 1999*).

1.8.2.ii Oxidative Stress and Endothelin Production

The effect of oxidative stress on cellular production of endothelin is not clear, with conflicting studies showing both upregulation and downregulation in a variety of cell culture systems. These results are summarised in table 1.6.

CELL CULTURE SYSTEM	SOURCE OF FREE RADICALS	EFFECT ON ENDOTHELINS	REFERENCE
Rat pulmonary artery cells	Glucose / glucose oxidase	↓ ET-1 mRNA ↓ ET-1	*Michael et al. 1997*
Bovine aortic endothelial cells	Hydrogen peroxide	↓ ET-1 mRNA ↓ ET-1	*Saito et al. 1998*
Human umbilical vein endothelial cells	Hydrogen peroxide Xanthine / xanthine oxidase	↑ ET-1 mRNA ↑ bigET-1 ↑ ET-1	*Kahler et al. 2000*
Human coronary artery smooth muscle cells	Hydrogen peroxide Xanthine / xanthine oxidase	↑ ET-1 mRNA ↑ bigET-1 ↑ ET-1	*Kahler et al. 2001*
Human aortic smooth muscle cells	Hydrogen peroxide	↑ ET-1 mRNA ↑ ET-1	*Ruef et al. 2001*

Table 1.6 The effect of oxidative stress on endothelin release

1.8.3 The S-nitrosothiols

As outlined above, S-nitrosothiols are long acting NO carriers, which will theoretically be generated as a result of nitrosative stress in conditions of high production of NO and ongoing oxidative stress, such as occurs in chronic liver disease. A major limitation to examining the role of S-nitrosothiols, however, is the lack of an effective assay for these compounds in biological samples.

1.8.3.i Assays for S-nitrosothiols

Although several assays have been described, the majority are performed in buffer solutions and cannot be extrapolated to blood or plasma. A general principle of biological assays is that one should be able to add the analyte to the biological fluid in question and demonstrate that the assay quantitatively measures an increment of the analyte concentration equal to the amount added (*Moore and Mani 2003*). Prior to this work no studies have been published that fulfil this criteria. The major problems encountered with assaying S-nitrosothiols include the relative instability of these compounds, trans-nitrosation reactions, the co-titration of other NOx species and the artificial generation of RSNOs during sample manipulation.

A method originally described for the quantification of thiols, the Saville assay (*Saville 1958*), has been adapted for the quantification of S-nitrosothiols. It is based upon the mercuric (Hg^{2+}) – catalysed release of nitrosonium ions (NO^{+}) from the S-nitrosated thiol, which then reacts with the aromatic amine, sulfanilamide to form a diazonium salt, followed by coupling to another aromatic amine, *N*-1-napthylethylethylenediamine HCl, which can be detected spectrophotometrically. The second step of the Saville reaction is therefore identical to the classical Griess assay for quantification of nitrite. Although this method can be used for the detection of RSNOs in simple aqueous solutions it is not sufficiently sensitive to detect endogenous RSNO levels in plasma. Furthermore, it depends on the measurement of differences between comparatively high levels of endogenous nitrite (which are subject to large inter- and intraindividual variation) and small incremental changes caused by the Hg^{2+}-catalysed liberation of nitrite from RSNOs.

Modifications of the original Saville method aimed at increasing sensitivity and separating the final reaction product from interfering nitrite have been described in combination with HPLC (*Akaike et al. 1997*) and sensitive fluorometric detection (*Cook et al. 1996, Park and Kostka 1997*). Concentrations as low as 10nM of individual low molecular weight RSNOs can be measured in physiological buffers by combining HPLC separation with either direct electrochemical detection or photolytic cleavage of RSNOs followed by chemiluminescent

detection of released NO (*Alpert et al. 1997*). However, these methods are limited by the sample volume that can be applied, which therefore limits sensitivity, and have only been described for the measurement of low molecular weight compounds such as S-nitrosocysteine and S-nitrosoglutathione. These, however, probably represent only a minor component of the total circulating pool, the majority of which is likely to exist as S-nitrosoalbumin.

1.8.3.ii Generation of S-nitrosothiols in vivo

Formation of RSNOs in human plasma was first suggested after administration of nitroglycerin by Fung *et al. 1988* who suggested that the vasorelaxant effects of organic nitrates may be mediated, at least in part, through the intermediate formation of RSNOs (*Chong and Fung, 1991*). The mechanisms by which RSNOs are formed *in vivo* is poorly understood. At physiological pH NO will not react directly with thiol groups (*Butler et al. 1995*), therefore generation has to be via an NO product.

On the basis of physiological experiments peroxynitrite has been proposed to be the nitrosating species responsible for S-nitrosothiol generation *in vivo.* When applied to aortic strips and pulmonary arteries peroxynitrie leads to vasorelaxation, the latter effect being dependent upon the presence of thiol groups in the form of glutathione (*Moro et al. 1995, Wu et al. 1994).* However, when peroxynitrite is added directly to thiols such as glutathione or albumin there is only nitrosation in the order of 1% (*Moro et al. 1994, Scorza & Minetti, 1998*). On the other hand simultaneous generation of superoxide and NO, resulting in *in situ* generation of peroxyitrite, leads to substantial thiol nitrosation (*Mayer et al. 1998, Schrammel et al. 2003*).

In the presence of oxygen NO can nitrosate thiols via the formation of intermediate nitrosating species such as $^{\bullet}NO_2$ or N_2O_3 (*Wink et al. 1994, Kharitonov et al. 1995*). This mechanism for the formation of S-nitrosothiols *in vivo* is highly controversial, with many investigators considering it to be too slow to lead to meaningful nitrosation reactions (*Gaston. 1999, Beckman and Koppenol, 1996*).

Nearly all the evidence cited above is based upon experimental work carried out using physiological buffers rather than biological matrices such as plasma, and therefore has to be interpreted with caution. The formation of S-nitrosothiols *in vivo* is likely to differ from experiments in physiological buffers, due to the difference in behaviour of NO/oxygen reactions in biological membranes (*Liu et al. 1998*) and the presence of metalloproteins such as caeruloplasmin *(Inoue et al. 1999*).

1.9 AIMS OF THIS THESIS

The central hypothesis to the work in this thesis is that oxidative stress and nitrosative stress effect the circulatory changes that occur as a part of advanced liver disease. These changes have important clinical consequences including variceal bleeding, development of ascites, the hepatopulonary syndrome and hepatic encephalopathy.

Due to local experience bile duct ligated rats were chosen as the experimental animal model. This model has the advantage of being associated with a high yield of cirrhosis and has been the focus of numerous previous studies which have shown that it is associated with oxidant injury and also increased production of nitric oxide.

The aims of the thesis were to confirm that cirrhotic liver disease is associated with increased production of isoprostanes, and then to extend previous studies on the vasoactivity of 8-iso-$PGF_{2\alpha}$ in renal models to the intrahepatic circulation of both normal and cirrhotic rats.

Whereas several studies have looked at the effect of antioxidant therapy on the progression of liver disease as judged by histology and biochemistry, none have examined the haemodynamic consequences of antioxidants in cirrhotic liver disease. For this reason lipoic acid treatment in rats with secondary biliary cirrhosis was chosen.

Theoretically S-nitrosothiols may be of importance in the hyperdynamic circulation of cirrhosis. Prior to the work in this thesis there have been no convincing quantifications of circulating plasma S-nitrosothiol levels, due to the significant limitations of the assay methods, outlined above. Therefore one of the main aims of this thesis was to develop an assay for plasma S-nitrosothiols which would be reproducible, sensitive and capable of examining a large quantity of samples.

CHAPTER 2 – MEASUREMENT OF ISOPROSTANE PRODUCTION IN HUMAN LIVER DISEASE

2.1 AIMS

In order to determine whether oxidant stress is a feature of liver disease both plasma and urinary isoprostane levels were measured and compared between four groups of individuals. 1) Childs C cirrhotic patients. 2) Childs A/B cirrhotic patients. 3) Patients with obstructive jaundice, and 4) Healthy controls.

In order to correct for variations in renal function creatinine clearance was also calculated so that isoprostane excretion could be expressed as a ratio of urinary isoprostane concentration to creatinine clearance.

2.2 METHODS

2.2.1 Subjects Studied

Ethics approval was obtained from The Royal Free Hospital ethics committee. Six normal controls were recruited from laboratory staff, mean age 31.2 (range 21 – 52). There were 6 patients with obstructive jaundice, confirmed by the presence of biliary dilatation on ultrasound. The aetiology was malignant obstruction in 5 (pancreatic cancer, n = 4, cholangiocarcinoma, n = 1) and extrinsic compression due to endometriosis in 1. All patients were studied prior to biliary decompression

A total of fifteen patients with cirrhosis were studied. Of these 6 had compensated liver disease with either Childs-Pugh stage A or B cirrhosis, mean age 53.0 (range 35 – 62) and mean Childs-Pugh score 7.0. A further nine patients had decompensated liver disease, with Childs-Pugh C stage cirrhosis, mean age 44.8 (range 36 – 51) and a mean Childs-Pugh score of 11.8. (*see table 2.1*)

2.2.2 Urine and Plasma Collection

Urine and plasma was collected from all the normal controls and the patients studied. The start and finish time of the urine collection was documented, and varied between 13 and 24 hours. Creatinine concentrations were measured in both plasma and urine, in order to calculate the creatinine clearance, as follows

$$\text{Creatinine Clearance} = \frac{\text{Urinary volume (ml)} \times \text{Urinary creatinine (mmol)}}{\text{Time (minutes)} \times \text{Plasma creatinine (mmol)}}$$

DIAGNOSIS	CHILDS-PUGH SCORE	CREATININE
Alcoholic Liver Disease	7	75
Alcoholic Liver Disease	7	79
Alcoholic Liver Disease	9	86
Alcoholic Liver Disease	7	73
Hepatitis C	6	87
Hepatitis C	6	100
Alcoholic Liver Disease	10	155
Alcoholic Liver Disease	13	533
Alcoholic Liver Disease	14	421
Alcoholic Liver Disease	12	198
Autoimmune Hepatitis	10	140
Autoimmune Hepatitis	12	191
Hepatitis C	11	152
Hepatitis C	11	119
Chronic Granulomatous Disease	13	143

Table 2.1 Cirrhotic patient details

2.2.3 Measurement of Isoprostanes

Measurement of both plasma and urinary isoprostanes was performed by gas chromatography mass spectroscopy (GCMS) following extraction, purification and derivatisation steps (*modified from the method first described by Morrow and Roberts 1994*).

In order to clean up the sample sufficiently to enable GCMS analysis a number of solid phase chromatography steps were performed. Solid phase cartridges (Waters, MA, USA) were used to remove water soluble and uncharged organic components. Thin layer chromatography steps were then used to further separate compounds

2.2.3.i Solid Phase Chromatography

One nanogram of deuterated D_4-8-iso-$PGF_{2\alpha}$ and 5ng of D_4-$PGF_{2\alpha}$ (Cayman Chemicals, MI, USA) were first added to 5ml pH 3.0 water as internal standards. Plasma or urine samples were then added and pH corrected to 3.0 with acetic acid. Initial extraction of the prostaglandin like compound was then performed using tC18 columns (-Si-$C_{18}H_{37}$) (tC18 Sep-Pak, Waters Corporation, MA, USA). These were prepared by washing with 6ml methanol followed by 6ml pH 3.0 water. The sample was then loaded onto the column and washed with 6ml pH 3.0 water to remove water soluble compounds. At this pH the isoprostanes remain relatively uncharged and are bound to the solid phase. The column was then washed with 6ml heptane to elute completely hydrophobic lipids. Isoprostanes were then eluted into polypropylene tubes with 6ml of heptane : ethyl acetate : methanol (40:50:10). These samples were then dried under nitrogen at 37°C in a water bath and resuspended in 80µl ethanol and then diluted with 3ml of ethyl acetate.

A further normal phase extraction was then carried out using a silica column (-Si-OH) (Waters Corporation, MA, USA). The cartridges were washed with 6ml ethyl acetate. The isoprostanes were then eluted with 6ml ethyl acetate:methanol (60:40) and dried under nitrogen.

2.2.3.ii Thin Layer Chromatography 1 (TLC)

Urine samples then underwent two additional clean up steps. Thin layer chromatography plates (Whatman Silica Gel 60A, Linear K6D, 5x20cm, 250μm thick) were pre-run in methanol for approximately 1 hour and then dried. Thin layer chromatography plates were prepared in advance with a mixture of chloroform, methanol, glacial acetic acid and water (93ml:7ml:1ml:0.8ml) and allowed to equilibrate for 1 hour. Samples were resuspended in 40 μl of chloroform:methanol (2:1), loaded onto the plates and run along with a separate plate loaded with 5μg $PGF_{2\alpha}$. The plates were run to ~ 13cm and dried. The $PGF_{2\alpha}$ plate was then dried, sprayed with 10% phosphomolybdic acid in ethanol and heated. This developed a band at between 5.5 and 6.5cm. The other plates were scraped at 1.5cm above and below the running position of $PGF_{2\alpha}$. The isoprostanes were then extracted with 900μl of ethylacetate : methanol (50:50)

2.2.3.iii Preparation of the Pentafluorobenzyl Ester

The pentafluorobenzyl ester was prepared by addition of 20μl 10% DIPEA (N,N – diisopropylethylamine) in acetonitrile and 40μl 10% PFBR (pentafluorobenzylbromide in acetonitrile). This was left at room temperature for 30 minutes and then dried down under nitrogen.

2.2.3.iv Thin Layer Chromatography 2

The pentafluorobenzyl esters were isolated following TLC using identical conditions to those described in the first TLC step, except using tanks prepared with a chloroform : ethanol mixture (93ml:7ml). On a separate plate 5 – 10μg of the isopropyl or methyl ester of $PGF_{2\alpha}$ was run as a standard. The solvent front was run to ~ 13cm above the application zone (~30 minutes). The PFB derivatives of $PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ ran at ~ 3.6 and 2.9cm respectively, with the methyl ester running just behind these compounds and the isopropyl ester (not shown below) running just in front. The sample plates were then scraped and the PFBR esters were extracted into 0.9ml ethyl nitrogen.acetate: methanol (50:50). The supernatant was removed after spinning and dried down under nitrogen.

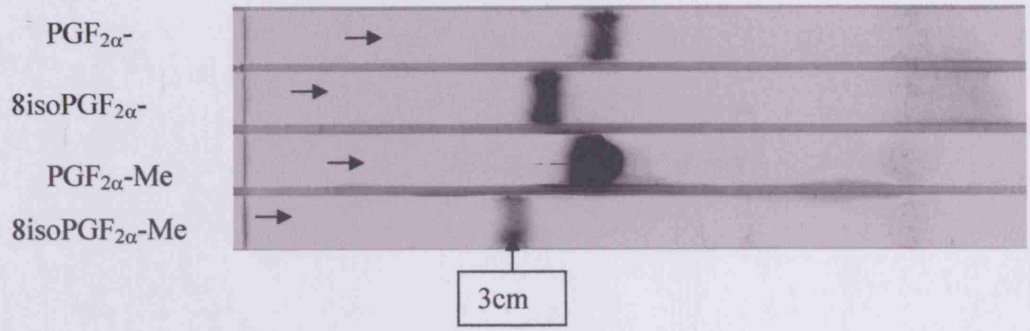


Figure 2.1 Thin layer chromatography showing the pentafluorobenzyl esters

2.2.3.v Preparation of the Trimethylsilyl Derivative

Final derivitisation was achieved by adding 10µl anhydrous dimethylformamide and 20µl BSTFA (bis-silyltrimethylfluouroacetamide) for 30 minutes under argon. Samples were dried down under nitrogen and resuspended in 10-20µl of undecane, transferred to a conical autosampler and sealed under argon.

2.2.3.vi Gas Chromatography - Mass Spectrometry

4µl of sample was injected into the GCMS. The isomers were seperated by gas chromatography with 8-iso-$PGF_{2\alpha}$ coming off the column first. Negative ion chemical ionisation with ammonia was used to determine the mass spectrum. Selective ion monitoring at 569 and 573 m/z was performed, as these were the masses of the predominant fragments of the undeuterated and deuterated forms of the isomers respectively.

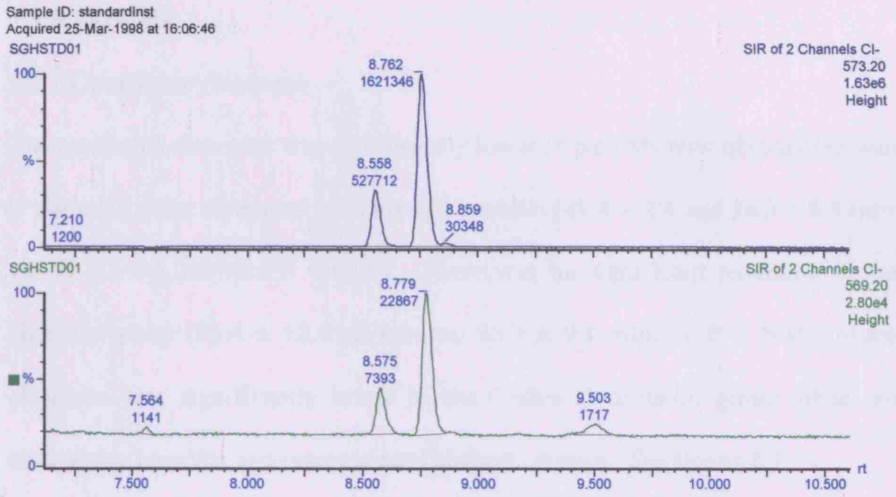


Figure 2.2 GC-MS trace of $PGF_{2\alpha}$,8-iso-$PGF_{2\alpha}$ and deuterated derivatives

$PGF_{2\alpha}$ retention time=8.779mins and 8-iso-$PGF_{2\alpha}$ (rt=8.575mins) at m/z 569.20 (B) and similar deuterated derivatives at 573.20 (A).

The concentration of isoprostanes in the sample was calculated from the ratio of the sample to the known concentration of the internal standard and expressed as pg/ml. In order to correct for variations in renal function, urinary isoprostanes are expressed as the ratio of the absolute concentration in urine divided by the creatinine clearance – pg/ml creatinine clearance.

2.2.4 Statistics

Comparison was made between the groups using a student t test, assuming unequal variances. A two tailed t test was employed with a P value of < 0.05 being considered as significant.

2.3 RESULTS

2.3.1 Creatinine clearance

The creatinine clearance was significantly lower in patients with obstructive jaundice and Childs C cirrhosis when compared with normal controls (49.4 ± 2.4 and 26.3 ± 6.8 ml/min respectively vs. 95.7 ± 9.1 ml/min. $P < 0.05$). There was no significant reduction in the Childs A + B cirrhotic group (83.4 ± 12.6 ml/min vs. 95.7 ± 9.1 ml/min. P = NS). Moreover, creatinine clearance was significantly lower in the Childs C cirrhotic group when compared to both obstructive jaundice and compensated cirrhotic groups. *See figure 2.3*

2.3.2 Plasma Isoprostane Concentration

Patients with Childs C cirrhosis had significantly higher levels of plasma isoprostanes when compared to normal controls (148.1 ± 35.5 pg/ml vs. 41 ± 3.1 pg/ml. $P < 0.05$). The levels in the obstructive jaundice and Childs A + B cirrhotic group were not significantly higher (69.3 ± 13.3 and 72.5 ± 13.5 pg/ml respectively vs. 41 ± 3.1 pg/ml. P = NS). *See figure 2.4.*

2.3.3 Urinary Isoprostane Concentration

When the urinary isoprostane concentration was corrected for creatinine clearance in individual patients, the levels were significantly higher in both the groups with Childs A + B and Childs C cirrhosis when compared with normal controls (80.9 ± 22.0 and 172.2 ± 51.8 pg/ml cr.cl respectively vs. 22.8 ± 2.5 pg/ml cr. cl. $P < 0.05$). There was no difference between the obstructive jaundice group and the normal controls (54.7 ± 13.4 pg/ml cr. cl vs. 22.8 ± 2.5 pg/ml cr. cl. P = NS). *See figure 2.5*

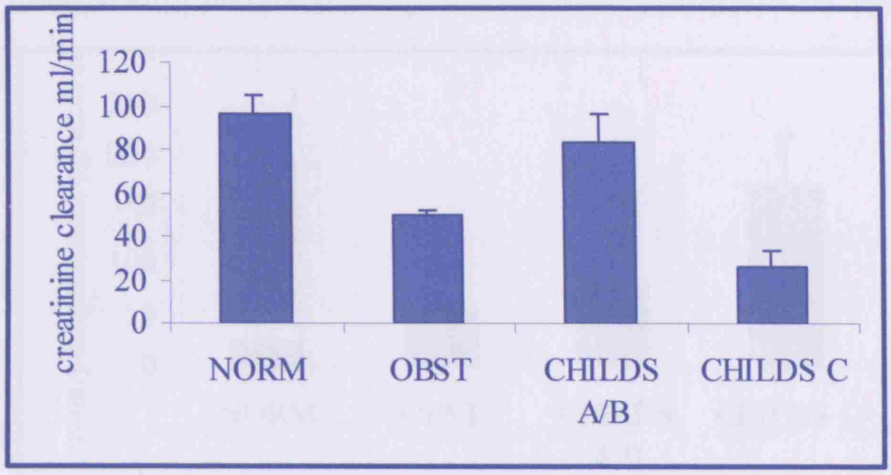


Figure 2.3 Creatinine clearance. The creatinine clearance was significantly lower in patients with obstructive jaundice and childs C cirrhosis when compared to normal controls. $P < 0.05$

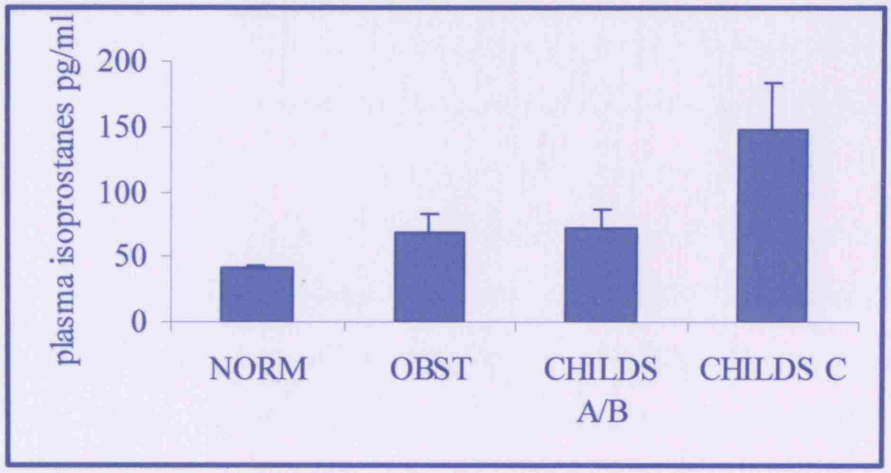


Figure 2.4 Plasma isoprostane levels. Plasma isoprostane levels were significantly higher in patients with Childs C cirrhosis when compared to normal controls $P < 0.05$

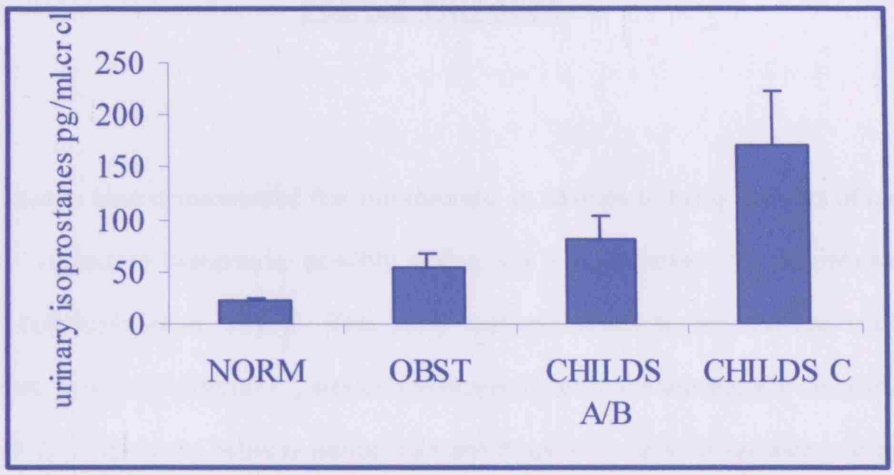


Figure 2.5 Urinary isoprostane excretion corrected for creatinine clearance. The levels were significantly higher in both Childs A + B and Childs C cirrhosis when compared with healthy controls. $P < 0.05$

CHAPTER 3 -THE EFFECTS OF ISOPROSTANES AND OXIDATIVE STRESS ON PORTAL PRESSURE

3.1 AIMS

Previous studies have demonstrated that isoprostanes, in addition to being markers of oxidative stress, are vasoactive componds, possibly acting via a thromboxane or thromboxane-like receptor (*Takahashi et al. 1992*). This study was performed to see whether one of the isoprostanes, 8-isoprostaglandin $F_{2\alpha}$ acts as a vasoconstrictor in the intrahepatic circulation, and to compare dose responses between normal rats and those with cirrhosis secondary to bile duct ligation. Moreover the effect of thromboxane receptor blockade on the response to isoprostane infusion was also examined.

A non-recirculating isolated perfused rat liver model was chosen, with a constant inflow rate. Changes in pressure were measured via a transducer in the portal vein and were assumed to represent vasoconstriction or vasodilatation at the level of the sinusoidal circulation or distal portal venous radicals.

In addition, it has been shown that ethanol infusion into normal rat livers leads to intrahepatic vasoconstriction, an effect that can be attenuated with endothelin antiserum (*Oshita et al. 1993*). The effect of ethanol infusion has never been compared between normal and cirrhotic rats. Moreover, as ethanol causes an increase in lipid peroxidation and production of F_2-isoprostanes (*Nanjii et al. 1994*), including 8-isoprostaglandin $F_{2\alpha}$ the role of both thromboxane and endothelin receptor blockade on the response to ethanol infusion was examined.

Previously endothelin and thromboxanes have been shown to cause stellate cell contraction. The effect of isoprostanes on this cell type has never been examined, so further studies looked at the effect of 8-isoprostaglandin $F_{2\alpha}$ on contractility of isolated stellate cells.

3.2 METHODS

3.2.1 Chemicals

8-iso-$PGF_{2\alpha}$, SQ29458, a thromboxane receptor antagonist, (7-[3-[[2-(phenylamino)carbonyl]-hydrazino]methyl]-7-oxabicyclo [2.2.2]hept-2-yl]-,[1S-[1α,2α,9Z),3α,4α]]-) and U46619, a thromboxane receptor agonist, (7-[-6-(3-hydroxy-1-octenyl)-2-oxabicyclo[2.2.1]hept-5-yl]-[1α,4α,5β9ZO,6α91E,3S*)]]-$C_{21}H_{34}O_4$ were purchased from Cayman Chemicals (Ann Arbor, Michigan, USA). Bosentan {4-tert-Butyl-N[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyrimidin-4-yl]-benzenesulfonamide sodium salt, a combined endothelin A and B antagonist, was a kind gift from Dr. Martine Clozel (Hoffman LaRoche, Basel, Switzerland). Pronase, DNAase and collagenase were purchased from Boehringer (Mannheim, Germany). Krebs-Henseleit buffer and all other reagents were purchased from Sigma Chemical Co. (Poole, Dorset, UK).

3.2.2 Bile Duct Ligation

Sprague-Dawley rats, 250 – 300g, were anaesthetised with intramuscular (i.m) hypnorm (Janssen Pharmaceuticals, Oxford,UK) and intraperitoneal (i.p) diazepam (Dumex Ltd, Tring, UK). After induction of anaesthesia the abdomen was shaved and a 1cm incision was made in the midline just beneath the costal margin. The duodenum was mobilised from its site beneath the liver with a small hook and the bile duct identified. The mesentery was divided either side of the bile duct and it was ligated with three separate sutures, two on the proximal duct and one distally. The bile duct was then cut between the proximal and distal sutures.

Fluid losses were replaced with 2 mls of sterile normal saline through the incision before the abdomen was sutured in two layers. Following recovery the animals were given free access to water and standard laboratory diet.

Animals were used between 24 and 28 days following bile duct ligation, by which stage macroscopic cirrhosis was evident. Microscopy confirmed massive ductular proliferation and architectural distortion with focal nodular formation (*see figure 3.1*). Bile duct ligated rats were

compared with a second group of animals, which had not undergone surgery, but were size matched. Between 4 and 9 animals were included in each group studied.

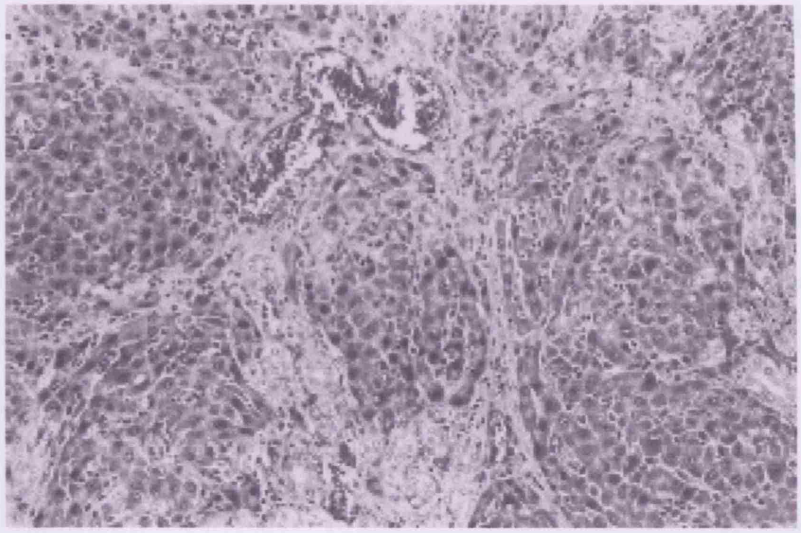


Figure 3.1 A representative histological section of rat liver with biliary cirrhosis. There is periportal ductular proliferation with focal portoportal and centroportal linkage, with some loss of hepatocytes.

3.2.3 Non-recirculating Liver Perfusion

Rats were anaesthetised with ether. The abdomen was opened via a longitudinal incision. After an intravenous bolus of 1000U of heparin via the inferior vena cava, the portal vein was cannulated (Abbocath 16G; Abbott Laboratories, Queensborough, UK) and the ascending inferior vena cava was severed. The liver was perfused in situ via a peristaltic pump (Watson-Marlow, Falmouth, UK) with Krebs-Hanseleit buffer (pH 7.4; 37°C) and saturated with 95% O_2 / 5% CO_2 at a constant rate of 25 mL/min; a damping reservoir made the flow non-pulsatile. When the baseline portal pressure was stabilised (5 – 10 minutes) , the compounds to be studied were coinfused with the perfusate (Harvard Infusion Pump, Harvard Instruments, South Natick, USA) at a rate that never exceeded an additional 500µL/min. The final concentrations of these compounds were determined by preliminary experiments, not included in the results. 8-iso-$PGF_{2\alpha}$ and U46619 were infused to achieve a final perfusate rate of 0.5, 1.0, 2.5, 50 and 10.0

nmol/min, for 2-3 minutes each. In the bile duct ligated cirrhotic rats, SQ29548 (5 nmol/min) was infused 5 minutes before commencing the infusion of 8-iso-$PGF_{2\alpha}$.

Ethanol was infused to achieve a final perfusate concentration of 25, 50, 75, 100, and 200 mM, for 3 minutes at each concentration. As with the isoprostane infusion experiments, in the bile duct ligated cirrhotic rats, SQ9548 (5 nmol/min) was infused 5 minutes before commencing the infusion of ethanol. To determine whether pre-treatment with an endothelin antagonist could block the response to ethanol, bosentan was infused for 5 minutes at a rate giving a final concentration of 10mg / 100ml, as previously described (*Rockey and Weisiger, 1996*), prior to commencing the infusion of ethanol.

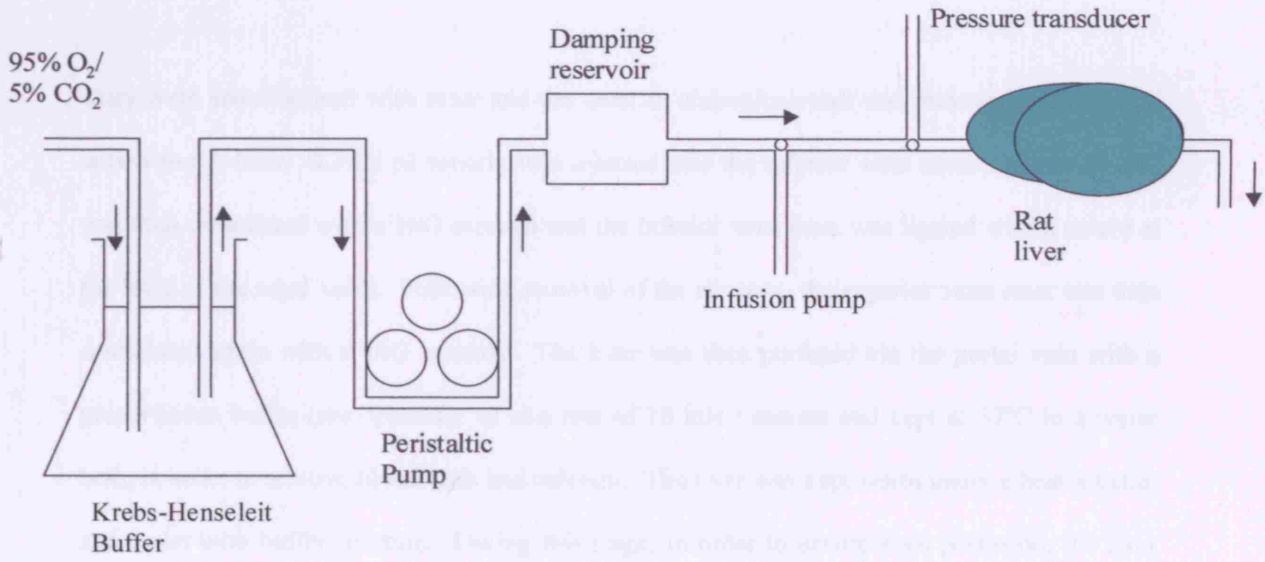


Figure 3.2 Isolated non-recirculating rat liver perfusion system

3.2.4 Portal Pressure Measurement

Portal pressure was monitored continuously via a pressure transducer connected to a three-way tap positioned proximal to the portal vein cannula. Measurements were performed using the MacLab system (ADInstruments, Hastings, UK). The value of portal pressure readings used for data analysis was taken as the highest response sustained for at least 30 seconds to each infused

rate, except when portal pressure decreased, in which case the trough pressure was used. In these case the lowest sustained response was measured.

3.2.5 Stellate Cell Isolation

This work was carried out with expert assistance, at The Liver Laboratory, University of California, San Francisco, under the supervision of Dr. Don Rockey.

3.2.5.i Liver Preparation

Retired breeder Sprague Dawley rats (weight ~ 800g) were used in order to increase the yield of cells. They underwent bile duct ligation as described above, and were used 14 days post-operatively.

They were anaesthetised with ether and the anterior abdominal wall was removed to allow full access to the liver. 0.3 ml of heparin was injected into the inferior vena cava. The portal vein was then cannulated with a 16G cannula and the inferior vena cava was ligated with a suture at the level of the renal veins. Following removal of the rib cage, the superior vena cava was then cannulated again with a 16G cannula. The liver was then perfused via the portal vein with a preperfusion buffer (*see appendix 4*) at a rate of 10 mls / minute and kept at 37°C in a water bath, in order to remove blood cells and calcium. The liver was kept warm under a heater lamp, and moist with buffer solution. During this stage, in order to ensure even perfusion, the liver was gently massaged with a cotton wool bud.

3.2.5.ii Enzymatic Digestion

After approximately 10 minutes the liver was then perfused with 100 mls of a high concentration pronase solution (*see appendix 4*) and then 250 mls of a combined collagenase and DNAase solution, again at 37°C. This enzymatic digestion continued for approximately 20 - 25 minutes after which the liver was removed from the animal and gently macerated in a suspension dish.

The liver was then placed in a siliconised bottle, to prevent cells from sticking. 100mls of a low concentration pronase solution (*see appendix 4*) was added to the solution and it was placed in a shaker water bath for 30 minutes at 37°C. The cell suspension was then strained through gauze and suspended in two 50 ml centrifuge tubes with Hams / Dulbecco MEM. 1ml of DNAase was added to each of the suspensions prior to centrifugation at 1,800 rpm for 7 minutes. The top 30 mls was discarded from each tube, as the stellate cells are found in the bottom 20 mls.

3.2.5.iii Isopyknic Centrifugation

The next separation technique involved density related ultracentrifugation. Two solutions of 15.6% and 8.2% nycodenz were prepared (*see appendix 4*), their optical densities were confirmed using a refractometer and they were sterilised through a 0.45 μM filter. 2.5 mls of the 15.6% solution was added to four ultracentrifuge tubes, and then 3mls of the 8.2% was carefully poured above this. 7.5 mls of the cell supernatant was added to each tube prior to centrifugation at 20,000 rpm for 25 minutes.

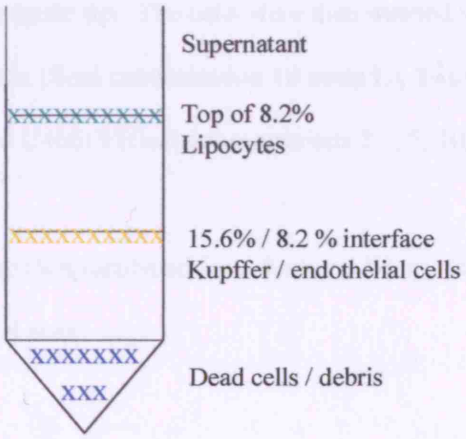


Figure 3.3 Cell distribution following ultracentrifugation

The stellate cell containing layer was aspirated and resuspended in Hams / Dulbecco MEM prior to two further centrifugation stages, both at 1,800 rpm and for 7 minutes. The final cell layer

was then resuspended in cell culture medium (*see appendix 4*) prior to performing the contractility assay.

3.2.6 Stellate Cell Contractility Assay

Contraction assays of stellate cells on collagen lattices were carried out according to the method of Rockey and Weisiger (*1996*). Flat bottomed 24 well tissue culture plates (Corning glass works, Corning, NY, USA) were used for the assay. They were pre-incubated with PBS containing 1% bovine serum albumin (0.5 ml / well) for 1 hour at 37°C, then washed with PBS and air dried. A 10 ml solution made up of 1 ml MEM buffer, 1 ml 0.2M HEPES and 8 mls collagen (vitrogen, sigma) was made up at 4°C and then pipetted into the wells (0.4 ml / well). The dishes were then incubated for 1 hour at 37°C to allow the solution to become gel-like. The stellate cells were then added to the wells and placed in a cell culture incubator for 24 hours, to allow adherence.

The collagen lattices were detached from the well plate by gentle circumferential dislodgement using a 200 µl micro-pipette tip. The cells were then washed with serum free medium alone, or medium with endothelin (final concentration 10 nmol/L), 8-iso-$PGF_{2\alpha}$ (final concentrations 2.5, 5, 10, 100 nmol/L) and U46619 (final concentrations 2.5, 5, 10, 100 nmol/L).

The culture plates were then incubated for a further 6 hours, and the contractility assessed as the percentage of initial gel area.

3.2.7 Statistics

Changes in portal pressure within groups were compared using a paired *t* test. To determine whether there was a statistically significant difference in the response for each compound infused between normal and cirrhotic rats, a Mann-Whitney *U* test was performed comparing the responses at each different dose in the two groups. Additional confirmation of the significant differential response was obtained using a Mann-Whitney *U* test comparing the

regression slope coefficient for each individual animal studied. P values of <0.05 were considered significant.

3.3 RESULTS

Infused dose (nmol/min)	Normal Rats (mmHg) portal pressure		BDL cirrhotic rats (mmHg) portal pressure	
	8-iso-$PGF_{2\alpha}$ (n=9)	U46619 (n=8)	8-iso-$PGF_{2\alpha}$ (n=9)	U46619 (n=6)
Baseline	8.2 ± 0.6	7.3 ± 0.5	12.0 ± 0.9	12.6 ± 1.0
0.5	8.1 ± 0.7	8.9 ± 1.1	13.9 ± 1.6	19.8 ± 3.2
1.0	8.7 ± 0.9	10.3 ± 1.9	17.5 ± 1.6	23.6 ± 3.5
2.5	9.8 ± 1.3	15.8 ± 2.1	18.6 ± 1.8	30.3 ± 4.3
5.0	10.3 ± 1.4	16.5 ± 1.7	19.7 ± 2.7	31.7 ± 5.0
10.0	10.7 ± 1.2	18.7 ± 1.3	20.0 ± 2.5	33.6 ± 4.2

Table 3.1 Mean portal pressure measurements following infusion of 8-iso-$PGF_{2\alpha}$ and U46619 in normal and cirrhotic rats. Values expressed as mean ± SEM.

3.3.1 Response of Portal Pressure to 8-iso-$PGF_{2\alpha}$ in Normal and Cirrhotic Rats

In preliminary control experiments, rat liver was perfused with Krebs-Hanseleit buffer. Portal pressure remained constant during a period of 60 minutes (n = 2). All further experiments were completed within this time.

In normal rats, there was a modest but significant increase in portal pressure after infusion of 8-iso-$PGF_{2\alpha}$. The portal pressure increased throughout the infusion with each incremental dose.

Mean portal pressure increased from a baseline value of 8.2 ± 0.6 to 10.7 ± 1.2 mmHg at the maximal dose infused (10.0 nmol/min; $P < 0.05$; $n = 9$).

In cirrhotic rats, baseline portal pressure was significantly higher than in normal rats (12.0 ± 0.9 vs. 8.2 ± 0.6 mmHg; $P < 0.05$), which was consistent with the development of portal hypertension. Infusion of 8-iso-$PGF_{2\alpha}$ caused a much greater increase in portal pressure in cirrhotic rats than in normal animals. Portal pressure increased in cirrhotic rats from a mean of 12.0 ± 0.9 to 18.6 ± 1.8 mmHg at a dose of 2.5 nmol/min, representing a change of mean portal pressure of 6.6 mmHg ($P < 0.05$; $n = 9$). At the same dose, the change of mean portal pressure in normal rats was 1.6 mmHg. At doses of > 2.5 nmol/min, the dose response curve plateaued with no significant increases observed above 2.5 nmol/min. Comparison of the magnitude of response to 8-iso-$PGF_{2\alpha}$ in normal rats vs. cirrhotic rats showed a statistically significant enhanced response in the cirrhotic rats ($P < 0.05$). (*see figure 3.4*)

3.3.2 Response of Portal Pressure to 8-iso-$PGF_{2\alpha}$ in the Presence of SQ29548

Infusion of the thromboxane receptor antagonist SQ29458 completely blocked the portal pressure increase induced by 8-iso-$PGF_{2\alpha}$ in cirrhotic rats. This experiment was not repeated in normal rats because the pressure increase was only modest. (*see figure 3.4*)

3.3.3 Response of Portal Pressure to U46619 in Normal and Cirrhotic Rats

Infusion of the thromboxane receptor agonist U46619 increased portal pressure in a dose-dependent manner in normal rats. Mean portal pressure increased from a baseline value of 7.3 ± 0.5 to 15.8 ± 2.1 mmHg at 2.5 nmol/min and to 18.7 ± 1.3 mmHg at the maximal dose infused (10 nmol/min; $P < 0.05$; $n = 8$).

As with 8-iso-$PGF_{2\alpha}$ there was an increased response to U46619 in the cirrhotic rats. Portal pressure increased from a mean value of 12.6 ± 1.0 to 30.3 ± 4.3 mmHg at a dose of 2.5 nmol/min, representing a mean increase in portal pressure of 17.7 mmHg in cirrhotic rats vs. 8.5 mmHg in normal rats at the same dose ($P < 0.05$; $n = 6$). The pressure continued to increase

with higher doses and reached 33.6 ± 4.2 mmHg at the maximal dose infused (10.0 nmol/min). As with 8-iso-$PGF_{2\alpha}$, when the dose response curves were compared, there was a significantly greater portal pressure response for each dose of U46619 infused in the cirrhotic group. (*see figure 3.5*)

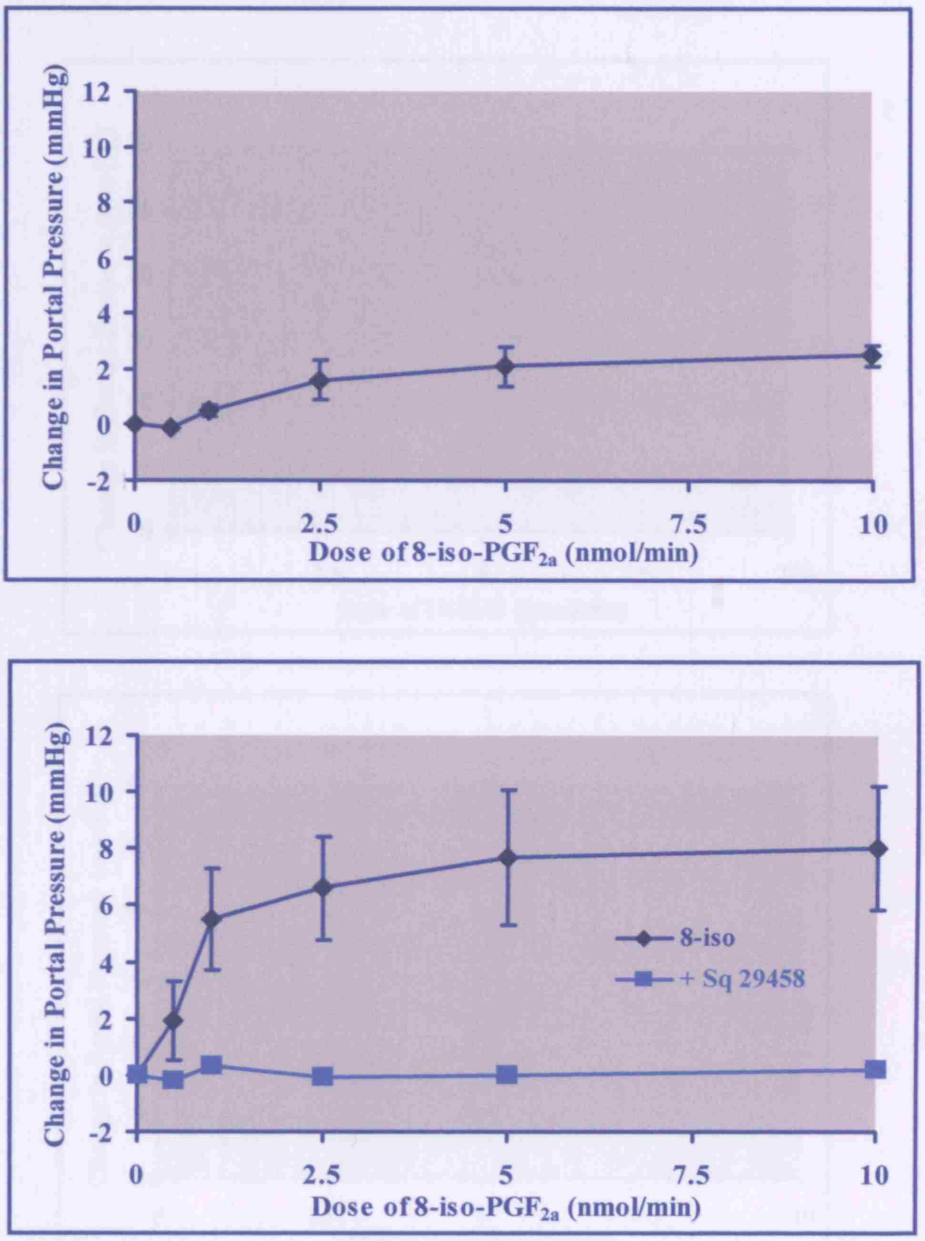


Figure 3.4 Dose-response curve of infusion of 8-iso-$PGF_{2\alpha}$, showing change in portal pressure with increasing doses in both normal (top graph – n = 9) and cirrhotic (bottom graph – n = 9) rats. Data are represented as the mean ± SEM. In cirrhotic rats the increase in portal pressure was completely blocked by co-administration of SQ29548, a thromboxane receptor antagonist (n = 6). The error bars are too small to be seen. When the responses in the two groups are compared, a significantly greater response is found in cirrhotic rats compared with normal rats ($P < 0.05$).

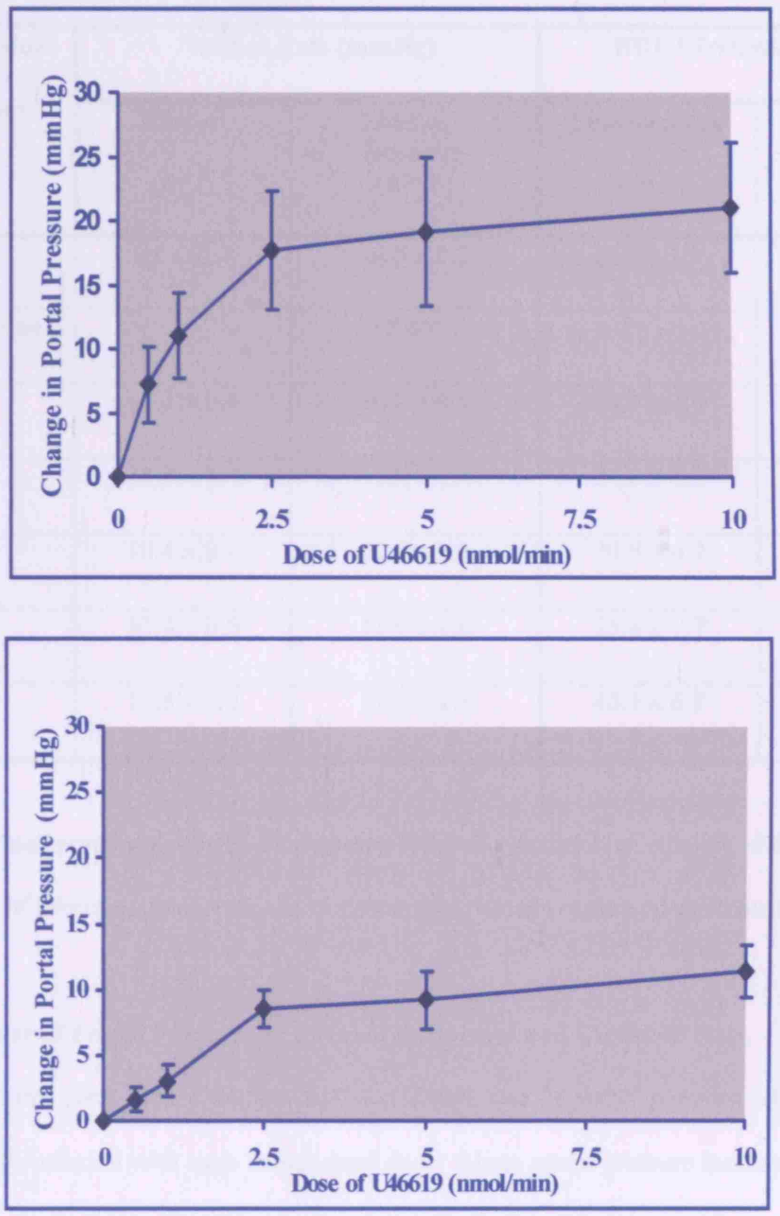


Figure 3.5 Dose-response curve of infusion of a thromboxane receptor agonist, U46619, showing changes in portal pressure with increasing doses in both normal (top graph – n = 8) and cirrhotic (bottom graph – n = 6) rats. Data are represented as the mean ± SEM. When the responses in the two groups are compared, a significantly greater response is found in cirrhotic rats compared with normal rats ($P < 0.05$).

Concentration of ethanol (mM)	Normal Rats (mmHg)		BDL Cirrhotic rats (mmHg)	
	Ethanol (n=7)	Ethanol + Bosentan (n = 8)	Ethanol alone (n = 9)	Ethanol + Bosentan (n = 8)
Baseline	9.0 ± 0.4	10.0 ± 0.7	16.6 ± 1.7	15.5 ± 2.5
Bosentan alone		9.8 ± 0.7		14.6 ± 2.5
25	10.3 ± 0.4	10.2 ± 0.9	20.0 ± 2.6	16.4 ± 3.3
50	10.9 ± 0.6	11.8 ± 1.4	24.6 ± 4.1	17.8 ± 4.6
75	10.4 ± 0.4	11.9 ± 1.1	30.5 ± 6.7	18.3 ± 5.0
100	11.4 ± 0.5	11.4 ± 1.0	35.8 ± 7.7	19.7 ± 5.1
200	13.8 ± 1.2	11.9 ± 1.1	45.1 ± 6.7	22.4 ± 6.6

Table 3.2 Mean portal pressure measurements following infusion of ethanol, either alone or in the presence of bosentan, in normal and cirrhotic rats. Values expressed as mean ± SEM.

3.3.4 Response of Portal Pressure to Ethanol in Normal and Cirrhotic Rats.

a) In normal rats there was a modest, but significant, rise in portal pressure which increased throughout the infusion with each incremental dose. Mean portal pressure increased from 9.0 ± 0.4 mmHg to 13.8 ± 1.2 mmHg, at the maximal dose infused (200mmol), $P< 0.05$ (*see figure 3.6*)

b) In bile duct ligated cirrhotic rats baseline pressure was significantly higher when compared with the normal controls (16.6 ± 1.7 vs 9.0 ± 0.4 mmHg, $P<0.05$) consistent with the development of portal hypertension. Ethanol infusion at the highest dose (200mM) caused a marked increase in portal pressure from a mean value of 16.6 ± 1.7 mmHg to 45.1 ± 6.7 mmHg ($P<0.005$), representing a six fold increase in the response when compared to normal rats (28.5 mmHg increase in cirrhotics vs 4.8 mmHg in normals) (*see figure 3.6*). There was a highly

statistically significant difference between the responsiveness of normal vs cirrhotic livers, $P<0.001$.

3.3.5 Response of Portal Pressure to Ethanol in the Presence of SQ29458

In cirrhotic animals co-infusion of the thromboxane receptor antagonist SQ 29548 did not lead to any significant reduction in the portal pressure response to ethanol (n = 4, *see figure 3.7*). The experiment was not repeated in normal rats.

3.3.6 Response of Portal Pressure to Ethanol in the Presence of Bosentan

a) In normal rats infusion of the endothelin receptor antagonist, bosentan, had no significant effect on the response to ethanol at the lower doses, however at the highest dose of ethanol the rise in portal pressure was blocked to a significant degree with the portal pressure increasing by 4.9 ± 0.9 mmHg vs 2.1 ± 0.6 mmHg, in the absence and presence of bosentan respectively $P < 0.05$ (*see figure 3.8*). When the overall response of the two groups of normal rats was compared the difference did not reach statistical significance, $p= 0.074$.

b) Infusion of bosentan caused a modest but significant decrease of baseline portal pressure (15.5 ± 2.5 vs 14.6 ± 2.5 mmHg, $P<0.05$) in cirrhotic rat livers. In contrast to normal controls the endothelin A / endothelin B receptor antagonist almost completely blocked the response to ethanol in 7 out of the 8 cirrhotic rat livers. In the presence of bosentan portal pressure increased by 7.8 ± 4.9 mmHg, at the highest dose of ethanol (200mM), compared with 28.5 ± 5.5 mmHg (no bosentan) (*see figure 3.9*). This increase at the highest dose was not statistically significant ($P=0.15$). There was a statistically significant inhibition of the response when the two cirrhotic groups were compared $P< 0.01$.

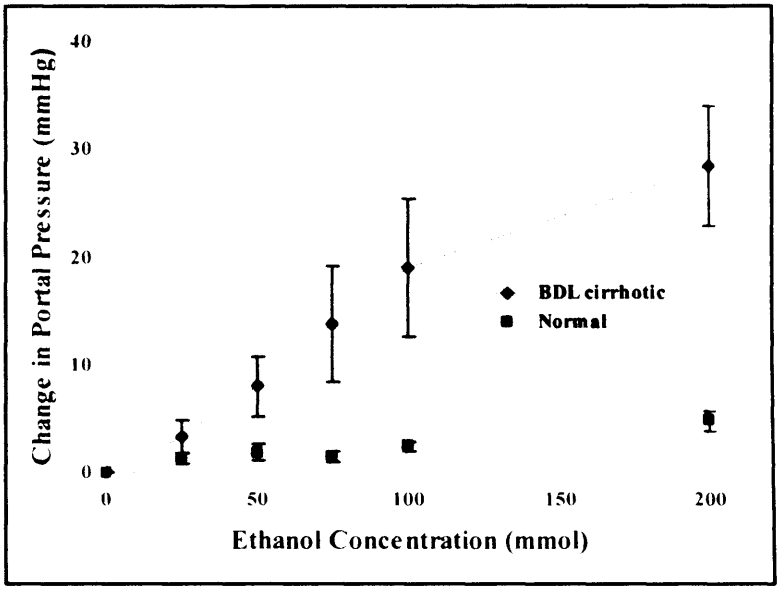


Figure 3.6 Dose response curves for the infusion of ethanol into isolated perfused rat livers from normal rats (n = 7) or rats which had undergone bile duct ligation (n = 9). Values are expressed as mean ± s.e.m. When the responses in the two groups are compared, a significantly greater response is found in cirrhotic rats compared with normal rats ($P < 0.05$).

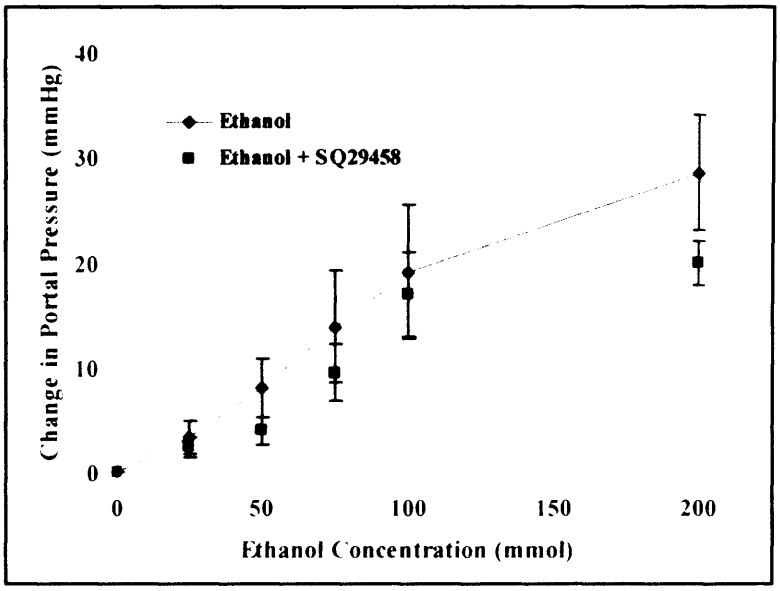


Figure 3.7 Dose response curve for the infusion of ethanol into cirrhotic rat livers either alone (n = 9) or in the presence of the thromboxane receptor antagonist SQ 29458 (n = 4). There was no significant difference between the responses.

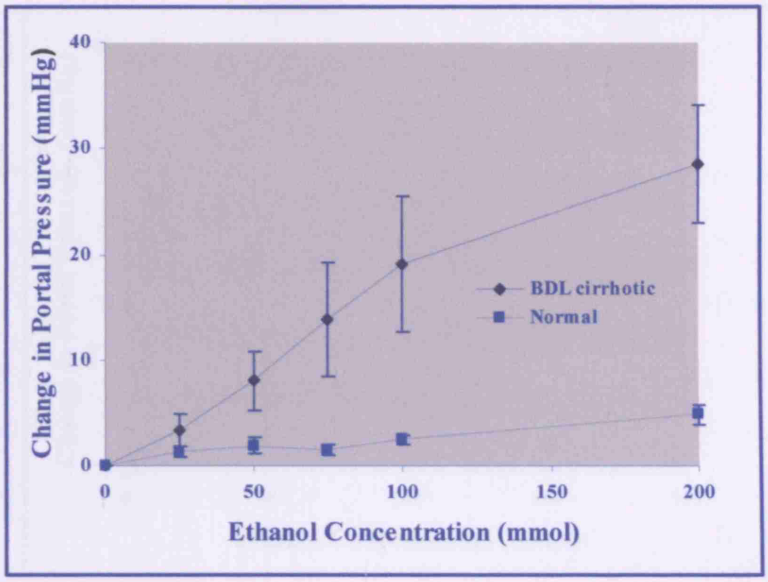


Figure 3.6 Dose response curves for the infusion of ethanol into isolated perfused rat livers from normal rats (n = 7) or rats which had undergone bile duct ligation (n = 9). Values are expressed as mean ± s.e.m. When the responses in the two groups are compared, a significantly greater response is found in cirrhotic rats compared with normal rats ($P < 0.05$).

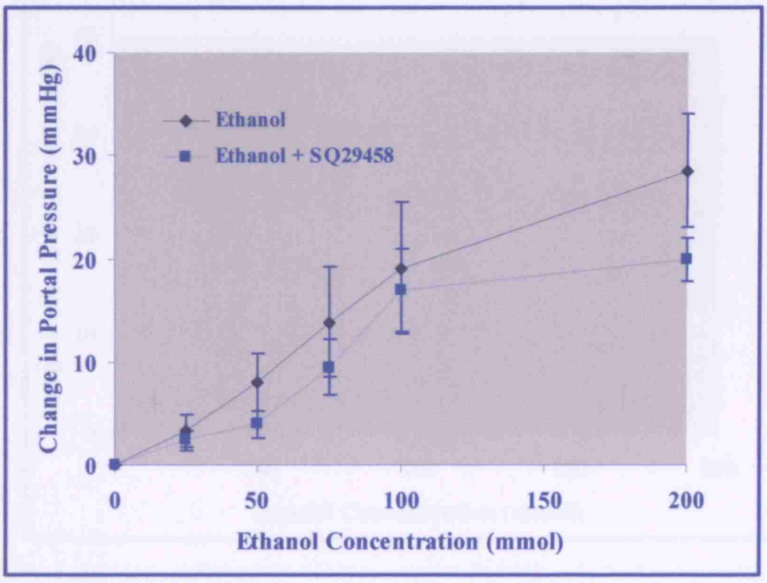


Figure 3.7 Dose response curve for the infusion of ethanol into cirrhotic rat livers either alone (n = 9) or in the presence of the thromboxane receptor antagonist SQ 29458 (n = 4). There was no significant difference between the responses.

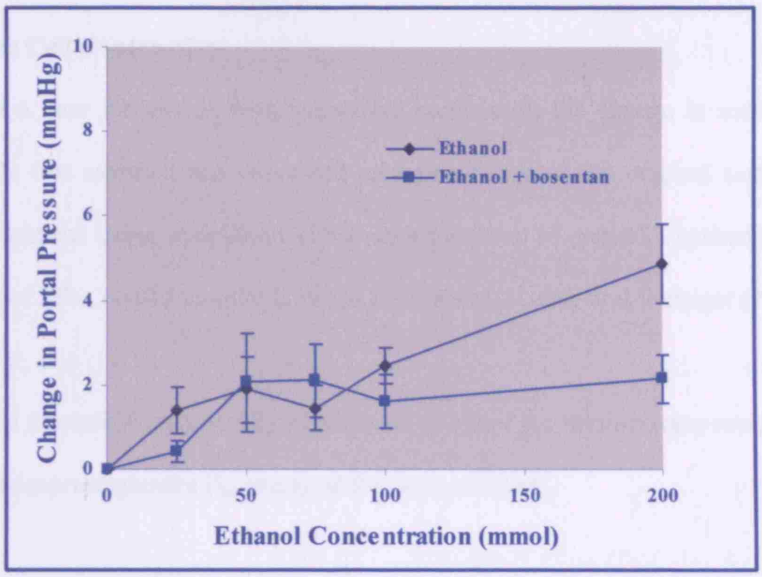


Figure 3.8 The effect of ethanol on portal pressure in normal rats either alone (n = 7) or in the presence of bosentan, a combined endothelin A and B receptor antagonist (n = 5). Values are expressed as the mean ± s.e.m. There is no significant difference between the responses.

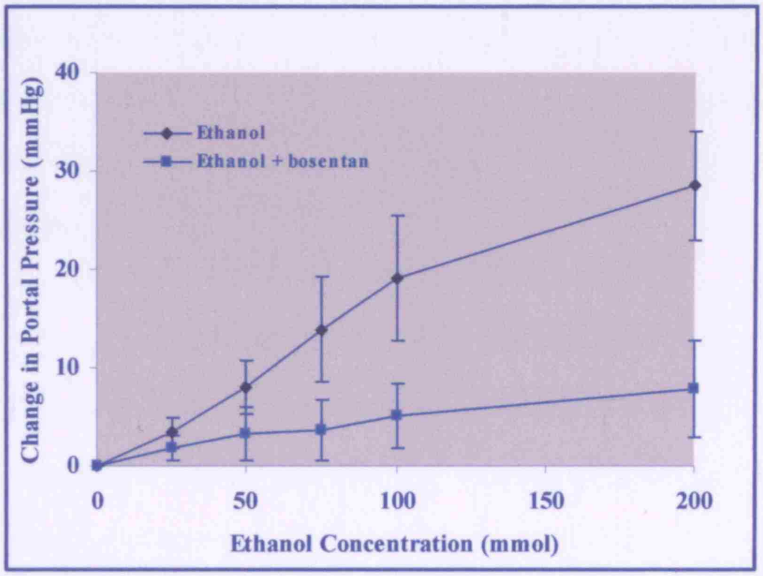


Figure 3.9 Dose responses to the infusion of ethanol alone (n = 9) or in the presence of the combined endothelin A and B receptor antagonist bosentan (n = 8) in bile duct ligated rats. Bosentan significantly inhibits the portal pressure response to ethanol (n = 8).

3.3.7 Stellate Cell Contractlity

Following a 6 hour incubation with vasoactive compounds the change in surface area of the collagen gels was assessed and expressed as a percentage of the original surface area. The control experiment using endothelin (final concentration 10 nmol/L) caused a reduction of surface area of 55%, confirming the previous findings of Rockey and Weisiger (*1996*).

There was no detectable contractility in response to either the thromboxane receptor antagonist, U46619 or 8-isoprostaglandin $F_{2\alpha}$ at any of the doses studied.

CHAPTER 4 – THE EFFECT OF LIPOIC ACID IN BILE DUCT LIGATED RATS

4.1 AIMS

The aim of this study was to determine whether chronic antioxidant therapy could prevent the development of the hyperdynamic circulation in bile duct ligated rats. In previous work (*Fernando et al. 1998*) the effect of the thiol containing antioxidant N-acetylcysteine has been investigated in the partial portal vein ligated model. In this work it was demonstrated that chronic administration of N-acetylcysteine for a period of 14 days significantly increased systemic vascular resistance and reduced cardiac index in this model as well as leading to a significant fall in portal pressure.

N-acetylcysteine is a difficult antioxidant to study chronically as it has to be administered twice daily by intraperitoneal injection. This is hazardous in animal models of cirrhosis due to the risks of introducing infection, particularly in the context of ascites, and of causing intraperitoneal bleeding. Lipoic acid, another thiol containing antioxidant, was chosen because of its high oral bioavailability, and hepatic pharmacodistribution. It has been advocated in the treatment of a variety of liver diseases, but has never been previously studied in relation to haemodynamic parameters.

The aim of this study was therefore to examine the haemodynamic consequences of chronic oral lipoic acid administration following bile duct ligation, and to compare this with a cohort of normal rats. In addition the effect on biochemistry, histology, nitric oxide production and lipid peroxidation was also examined.

4.2 METHODS

4.2.1 Experimental Animals

All animal experiments were performed in accordance with local guidelines. Male Sprague-Dawley rats (250-300g) were used. Sixty eight animals were housed in the Comparative

Biology Unit on a normal rodent chow diet (expanded SDSRMI, Witham, UK), with a light cycle of 12 hours on and 12 hours off, at a temperature of 19°C to 23 °C and humidity of ≅ 50%. 34 animals underwent bile duct ligation, and 34 rats were used as controls. Following anaesthesia with intramuscular hypnorm (Janssen Pharmaceuticals, Oxford, UK) and intraperitoneal diazepam (Dumex Ltd, Tring, UK), a midline laparotomy was performed, the bile duct was localised, triply ligated and then cut. The bile duct ligated animals either underwent haemodynamic evaluation or were used for plasma and tissue collection 24 days following surgery.

Lipoic Acid. Because lipoic acid is insoluble in water it was converted to its sodium salt by adding the minimum volume of 1 mol/L sodium hydroxide required to cause complete dissolution, and was then back titrated to pH 7.0 with 0.1 mol/L hydrochloric acid. The presence of lipoic acid in the drinking water had no effect on the total volume consumed by either the BDL or normal rats. The rats were divided as below.

BDL Group: The rats were divided into two groups. The first group (n = 17) was given free access to drinking water with lipoic acid added (1g/L), and a second group (n = 17) was given free access to drinking water alone.

Normal Group: The rats were divided into two similar groups (n = 17 each group).

Haemodynamic studies. These were performed on day 24 after surgery, in the BDL group, or a similar period in the control group, under sodium pentobarbitone anaesthesia (60 mg/kg intraperitoneally). Mean arterial pressure (MAP) and portal pressure (PP) were determined by direct cannulation of the right femoral artery and the ileocolic vein, respectively, with 22-gauge Abbocaths (Abbott Laboratories, Kent, UK), and the pressure recordings were monitored by a MacLab computer (ADInstruments, Sussex, UK). All pressure transducers were calibrated immediately before each recording, with the zero reference point being the midportion of the rat. The cardiac output was measured by thermodilution using AB6B2-B05-type thermistors

(Thermometrics, Edison, USA) bonded by epoxy resin into a 22-gauge 10 cm polyvinyl chloride single lumen catheter (PVB, Kircheeson, Germany). The intra- and intercardiac output coefficients of variation for the thermistors were 2.9% ± 0.7% and 4.9% ± 0.4%, respectively, as determined by using a cardiac output simulator. In brief, a thermistor was passed into the carotid artery and advanced to the aortic arch. A thermal indicator (0.1 mL of normal saline at room temperature) was then injected into the right atrium via an 8 cm length of PE-50 tubing placed in the jugular vein. The core temperature of the rat was maintained at 37 ± 0.5°C. A typical thermodilution curve had a rapid upstroke and a slow decay, and at least three thermodilution curves were obtained for each rat. Curves with unusual morphology were ignored. The cardiac output ($mL.min^{-1}$) was calculated using the arithmetic mean of the computed integral. The systemic vascular resistance index (SVRI) was calculated as mean arterial pressure (MAP)/ cardiac index.

Sample collection. Tissue and blood were harvested 24 days following either bile duct ligation or lipoic acid supplementation, following anaesthesia with intramuscular hypnorm (Janssen Pharmaceuticals, Oxford, UK) and intraperitoneal diazepam (Dumex Ltd, Tring, UK). No rats were used for both haemodynamic studies and sample collection. Liver tissue was immediately snap frozen in liquid nitrogen and stored at –70°C prior to the enzyme and glutathione assays. A second piece of tissue was stored in formalin prior to preparation of slides. Blood was collected into tubes containing ethylenediaminetetraacetic acid (EDTA) and centrifuged for 10 minutes at 3,500g. The plasma aliquot was stored at –70°C prior to being assayed.

4.2.2 Biochemistry and Histology

Aspartate transaminase, albumin, bilirubin and creatinine were measured using an auto-analyzer (Hitachi, Tokyo, Japan). Haematoxylin-eosin slides of liver were prepared and assessed by a histopathologist, who was blinded as to the treatment the rats had received. The slides were scored on a scale of 0 to 8, on the basis of fibrosis (0 – 2), biliary proliferation (0 - 3) and inflammatory infiltrate (0-3).

4.2.3 Plasma Nitrite and Nitrate Assay

Plasma nitrite and nitrate were measured using a chemiluminescense NO analyzer (Sievers Research Inc, Boulder, USA) (*see section 5.2.3*). Plasma was filtered through 30,000 molecular weight cut-off filters (Millipore, Bedford, USA), to prevent foaming secondary to plasma proteins in the reaction chamber. Samples were incubated for 1 hour at 37°C with nitrate reductase, flavine adenine dinucleotide and nicotinamide adenine dinucleotide to convert nitrate to nitrite. Samples were then injected into a reaction chamber containing acetic acid and potassium iodide (50 mg/mL) at a ratio of 4:1. This reduces nitrite to NO, which is purged from the refluxing solution by nitrogen and reacts with ozone before analysis by chemiluminescense. Measurements were calibrated against standard curves of sodium nitrite and sodium nitrate, which had also been incubated with nitrate reductase. Using this method there was a conversion rate of nitrate to nitrite of greater than 90%.

4.2.4 Hepatic Nitric Oxide Synthase Activity Assay

Total nitric oxide synthase (NOS) activity was determined by the conversion of ^{14}C-arginine to ^{14}C-citrullline using a commercial kit (Stratagene, La Jolla, USA).

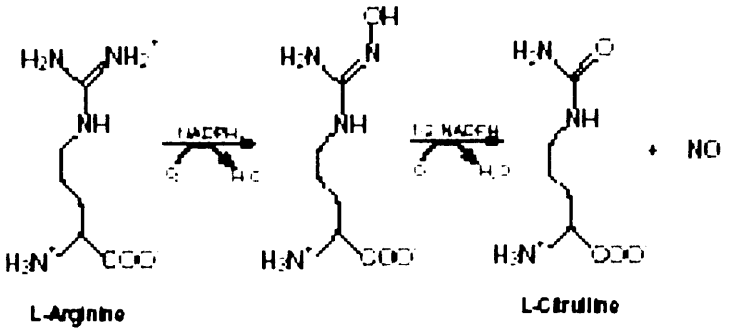


Figure 4.1 Arginine – citrulline assay

Liver tissue was homogenised in a buffer containing ethylenediaminetetraacetic acid (EDTA) and ethyleneglycol-bis(ß-aminoethyl ether)-N.N.N'.N'-tetraacetic acid and kept at 4°C. The homogenate was centrifuged and 10 µL of the supernatant was incubated with a reaction buffer

containing tetrahydrobiopterin, flavine adenine dinucleotide, flavine adenine mononucleotide, reduced nicotinamide adenine dinucleotide phosphate, calcium chloride and ^{14}C-arginine for 30 minutes at room temperature. The reaction was stopped using a buffer containing HEPES 9 (pH 5.5), EDTA and equilibrated Dowex resin (AG 50W-8). The EDTA chelates the calcium required by NOS and consequently inactivates the enzyme. The resin binds arginine, but not citrulline, which is ionically neutral at pH 5.5. Therefore following centrifugation, the supernatant contains ^{14}C-citrulline, but not ^{14}C-arginine, which was measured in a liquid scintillation analyser with automatic quench correction (Kontron, Watford, UK). The radioactivity was compared against a blank provided within the kit. Protein content of the homogenate supernatant was determined using the Bradford reagent, and total NOS activity was expressed as nanomoles of ^{14}C-arginine converted to ^{14}C-citrulline per milligram of protein per minute.

4.2.5 Lipid Peroxide Assay

The lipid peroxidation products, malonaldehyde and 4-hydroxyalkenals were assayed using a commercial kit (Oxis International, Portland, USA). The assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with malonaldehyde and 4-hydroxyalkenals at 45°C, which yields a stable chromophore that is detected spectrophotometrically at 586 nm.

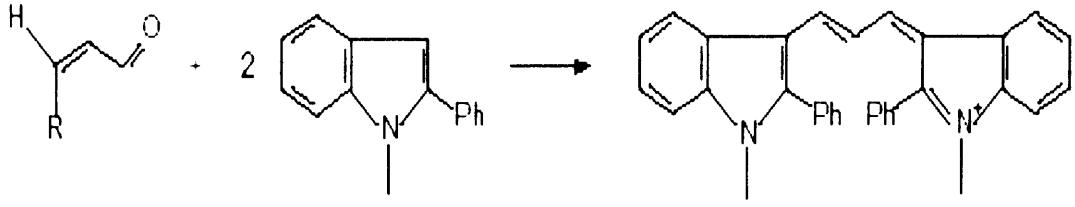


Figure 4.2 Lipid peroxide assay

200 µl aliquots of plasma were added to 650 µl of N-methyl-2-phenylindole in acetonitrile, vortexed and 150 µl of methane sulfonic acid was added. The samples were incubated in a water bath at 45°C for 60 minutes. The samples were then centrifuged to obtain a clear

supernatant (15,000g for 10 minutes) and the absorbance was measured at 586 nm. Samples were compared against a standard curve of malondialdehyde and an appropriate blank using methanol/acetonitrile and plasma. The molar extinction coefficient was approximately 120,000.

4.2.6 Glutathione Assay

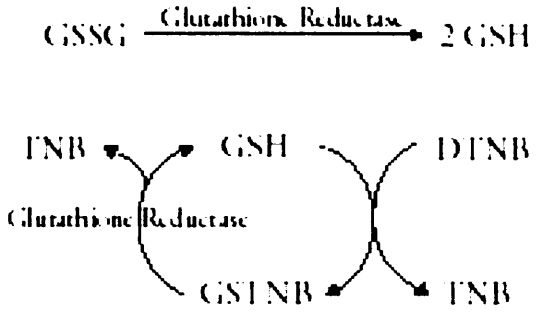


Figure 4.3 Assay for reduced glutathione

Reduced glutathione was measured by a spectrophotomeric assay using Ellmans reagent and an enzymatic recycling step. Tissue was homogenised in 3% sulphosalicylic acid to give a 10% wt/vol tissue homogenate. The homogenate was mixed with either n-ethylmaleimide (NEM) to measure oxidised glutathione, or hydrochloric acid to measure total glutathione. This was then centrifuged for 4 minutes at 14,000 rpm, and the supernatant was passed through a methanol-activated C_{18} cartridge (Waters, Milford, USA) and was eluted with potassium phosphate buffer (pH 7.2). The effluent was then added to a 1.2 mmol/L solution of 5,5'-dithiobis-2-nitrobenzoic acid (Ellman's reagent) in potassium phosphate buffer (pH 7.2). Finally, the solution was incubated for 10 minutes at room temperature with a solution containing 100 mmol/L imidazole, 0.5 mg nicotinamide adenine dinucleotide per millilitre and 1.6 U glutathione reductase per millilitre. The sulfhydryl group of GSH reacts with Ellman's reagent to produce a yellow coloured 5-thio-2-nitrobenzoic acid (TNB). The mixed disulphide, GSTNB (between GSH and TNB) that is concomitantly produced, is reduced by the glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly

proportional to this recycling reaction, which in turn is directly proportional to the concentration of GSH in the sample.

Absorption was measured at 412 nm and reduced glutathione was calculated as the difference between parallel samples initially treated with hydrochloric acid and NEM. A standard curve of glutathione was used to calculate concentrations.

4.2.7 Statistics

All of the observations are reported as mean ± SEM. Statistical analysis was performed using one-way ANOVA and pairwise multiple comparison procedures by Duncan's method. $P < .05$ was regarded as statistically significant.

4.3 RESULTS

4.3.1 Haemodynamic Measurements

Normal Versus BDL

There was a significant increase in CI in rats with BDL cirrhosis when compared with the normal group (29.5 ± 2.1 vs. 15.8 ± 0.6 $ml.min^{-1}.100g^{-1}$, BDL vs. normal, respectively; $P < .05$). This was accompanied by a reduction in SVRI (3.7 ± 0.4 vs. 7.2 ± 0.2 $mmHg.mL^{-1}.min.100g$; $P < .05$), but no significant difference in mean arterial pressure (104 ± 3.6 vs. 114 ± 3.1 mmHg; P = NS). PP increased following bile duct ligation (15.2 ± 0.5 vs. 8.6 ± 0.4 mmHg; $P < .05$)

BDL + Lipoic Acid Versus BDL

Chronic administration of lipoic acid prevented the development of the hyperdynamic circulation in biliary cirrhotic rats. CI was significantly lower in the lipoic acid group (15.7 ± 2.0 vs. 29.5 ± 2.1 6 $ml.min^{-1}.100g^{-1}$; $P < .05$) and the systemic vascular resistance was higher (7.3 ± 0.9 vs. 3.7 ± 0.4 $mmHg.mL^{-1}.min.100g$; $P < .05$). In addition, PP was significantly lower in the lipoic acid treated group (12.7 ± 0.8 vs. 15.2 ± 0.5 mmHg; $P < .05$). There was, however, no significant change in MAP (109 ± 5.5 vs. 104 ± 3.6 mmHg; P = NS).

Normal + Lipoic Acid Versus Normal

Lipoic acid had no significant effect on CI, SVRI, MAP or PP in normal animals. (CI: 14.6 ± 1.6 vs. 15.8 ± 0.5 $ml.min^{-1}.100g^{-1}$, SVRI: 9.0 ± 1.5 vs. 7.2 ± 0.2 $mmHg.mL^{-1}.min.100g$; MAP: 120 ± 4.5 vs. 114 ± 3.1 mmHg; PP: 8.4 ± 0.6 vs. 8.5 ± 0.4; P = NS).

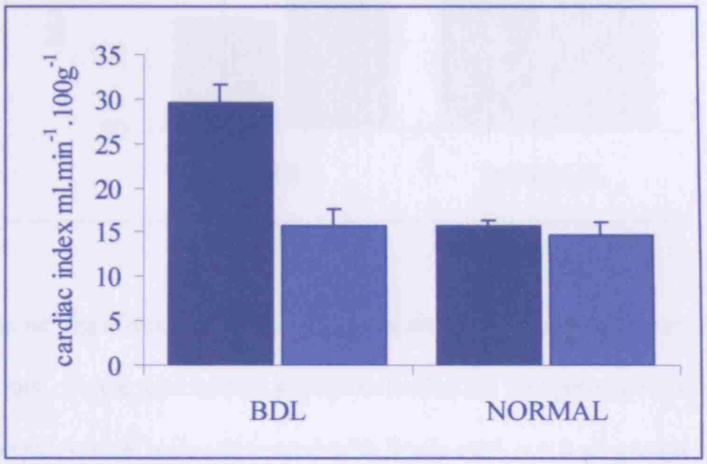


Figure 4.4 Cardiac index is significantly increased by bile duct ligation. In rats with secondary biliary cirrhosis this increase is prevented by administration of lipoic acid. Solid bars indicate the rats which were not treated with lipoic acid. n = 8 all groups.

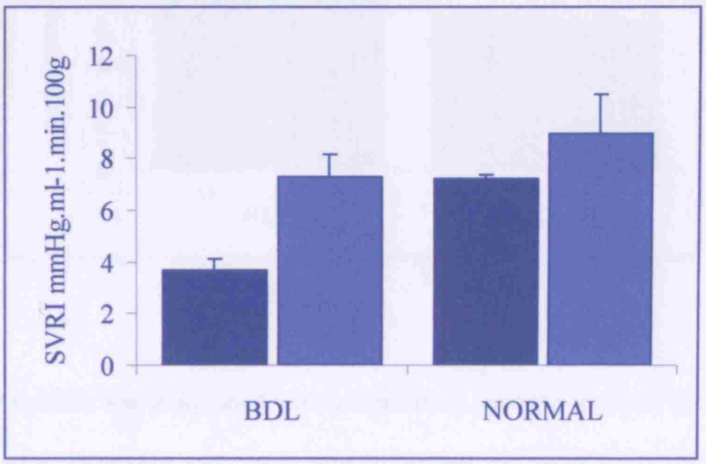


Figure 4.5 The systemic vascular resistance index is significantly lower in rats with secondary biliary cirrhosis by comparison with normal rats. Treatment with lipoic acid prevents this decrease. Solid bars indicate the rats which were not treated with lipoic acid. n = 8 all groups.

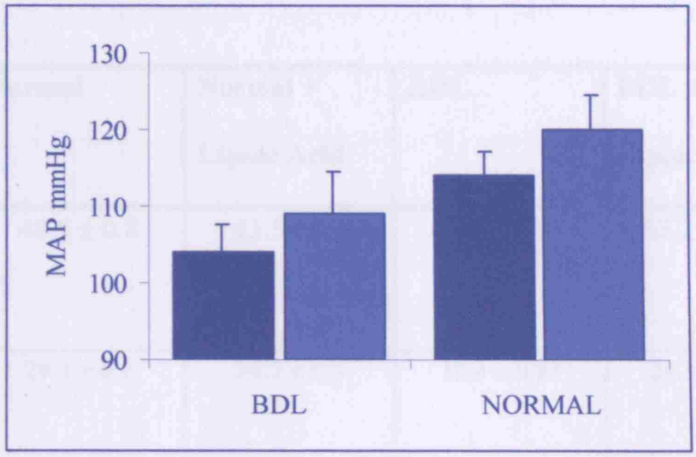


Figure 4.6 There was no significant difference in mean arterial pressure between the untreated cirrhotic and normal rats. Lipoic acid had no significant effect on this parameter in either group. Solid bars indicate the rats which were not treated with lipoic acid. n = 8 all groups.

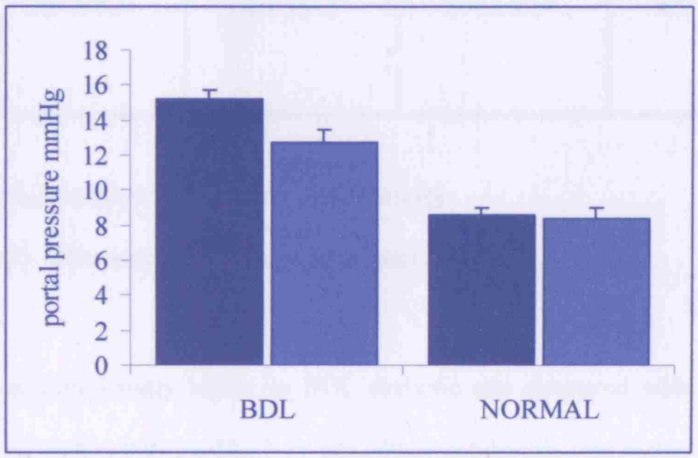


Figure 4.7 Bile duct ligation was associated with a significant increase in portal pressure. This increase was prevented by treatment with lipoic acid. Solid bars indicate the rats which were not treated with lipoic acid. n = 8 all groups.

4.3.2 Biochemistry and Histology

	Normal	Normal + Lipoic Acid	BDL	BDL + Lipoic Acid
Creatinine μmol/l	48.5 ± 0.8	43.5± 0.3	62.4 ± 2.2*	63.2 ± 3.1*
Albumin g/l	29.3 ± 0.2	28.7 ± 0.3	22.4 ± 0.8*	24.4 ± 0.8*
Bilirubin μmol/l	2.0 ± 0.8	2.0 ± 1.0	122.9 ± 5.9*	127.8 ± 14.4*
AST IU/l	101 ± 23.4	71 ± 2.1	426 ± 28.2*	385 ± 84.4*
HISTOLOGY SCORE	1.0 ± 0.3	0.9 ± 0.3	6.4 ± 0.1*	6.2 ± 0.2*

Table 4.1 Effect of lipoic acid on biochemistry and histology

* indicates significantly different results from normal rats ($P < 0.001$)

Plasma creatinine was significantly higher in BDL cirrhotic rats compared with normal rats (62.4 ± 2.2 μmol/L vs. 48.5 ± 0.8 μmol/L; $P < .05$). Plasma bilirubin was higher in the BDL cirrhotic group (123 ± 6 μmol/L vs. 2.0 ± 0.8 μmol/L; $P < .05$), confirming biliary obstruction. Furthermore, aspartate transaminase was higher (426 ± 28.2 U/L vs. 101 ± 101 ± 23.4 U/L; $P < .05$), and albumin was lower (22.4 ± 0.8 g/L vs. 29.3 P 0.2 g/L; $P < .05$) in the cirrhotic group of animals. Lipoic acid had no significant effect on any of these parameters in either group. Histological examination confirmed the development of biliary cirrhosis in the BDL group, and this too was unaffected by the administration of lipoic acid.

4.3.3 Plasma Nitrite / Nitrate

Plasma nitrite / nitrate concentrations were measured as an indirect index of NO production. In the BDL cirrhotic rats, there was a significant increase in the combined nitrite and nitrate levels (40.3 ± 2.7 vs. 29.4 ± 2.2 μmol/L; P < .05). Lipoic acid had no effect on nitrite and nitrate in normal rats (26.1 ± 2.2 vs. 29.4 ± 2.2 μmol/L; P = NS), but significantly decreased levels in the BDL cirrhotic group (33.4 ± 1.3 vs 40.3 ± 2.7 μmol/L; P < .05). See Figure 4.8

4.3.4 Hepatic NOS Activity

Total hepatic NOS activity was significantly increased in the livers from BDL rats compared with normal controls (4.27 ± 0.4 vs. 2.25 ± 0.2 $nmol.mg^{-1}.min^{-1}$; P < .05). This increase was significantly attenuated by the administration of lipoic acid to the BDL cirrhotic rats (3.29 ± 0.1 vs. 4.27 ± 0.4 $nmol.mg^{-1}.min^{-1}$; P < .05) but there was no significant effect in normal controls (2.10 ± 0.1 vs. 2.25 ± 0.2 $nmol.mg^{-1}.min^{-1}$; P = NS). See Figure 4.9

4.3.5 Plasma Lipid Peroxides

Two products of lipid peroxidation, namely malonaldehyde and 4-hydroxyalkenals, were measured in an assay that measures these combined reactive aldehydes. Following bile duct ligation, plasma concentration of these combined lipid peroxides increased from 5.4 ± 0.9 μmol/L to 8.1 ± 0.9 μmol/L (P <.05) for normal and BDL rats, respectively. Lipoic acid decreased the concentration of lipid peroxides in the normal rats (2.4 ± 0.8 μmol/L vs. 5.4 ± 0.9 μmol/L; P<.05), but had no effect on plasma concentrations in the BDL group (7.1 ± 0.7 μmol/L vs. 8.1 ± 0.9 μmol/l; P = NS). See Figure 4.10

4.3.6 Hepatic Levels of Reduced Glutathione

The hepatic level of reduced glutathione was decreased in the BDL cirrhotic group (1.96 ± 0.2 vs. 2.8 ± 0.5 μmol/g tissue in the BDL and normal controls, respectively), but this was not statistically significant. Lipoic acid significantly increased the concentration of reduced glutathione in the BDL group (1.96 ± 0.2 to 5.1 ± 0.8 μmol/g tissue). Hepatic levels of

glutathione also increased in normal controls (2.8 ± 0.5 to 4.1 ± 0.2 μmol/g tissue), but this did not reach statistical significance (P >.05). See figure 4.11

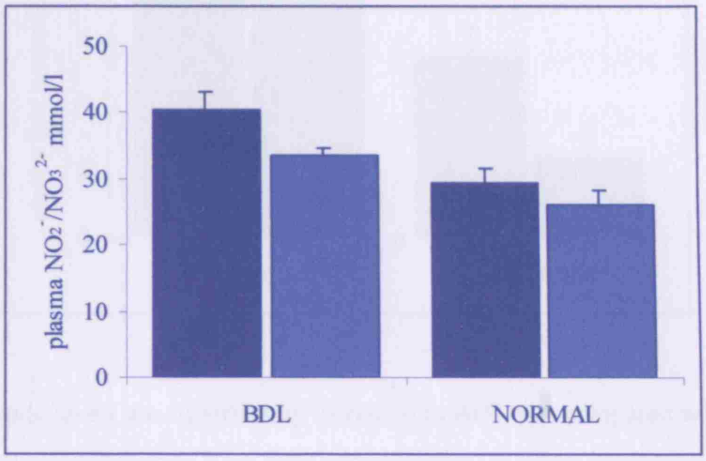


Figure 4.8 Plasma nitrite and nitrate levels are increased in rats with biliary cirrhosis. Lipoic acid treatment significantly attenuated this increase but had no effect on normal rats. Solid bars indicate rats which were not treated with lipoic acid. n = 9 for all groups.

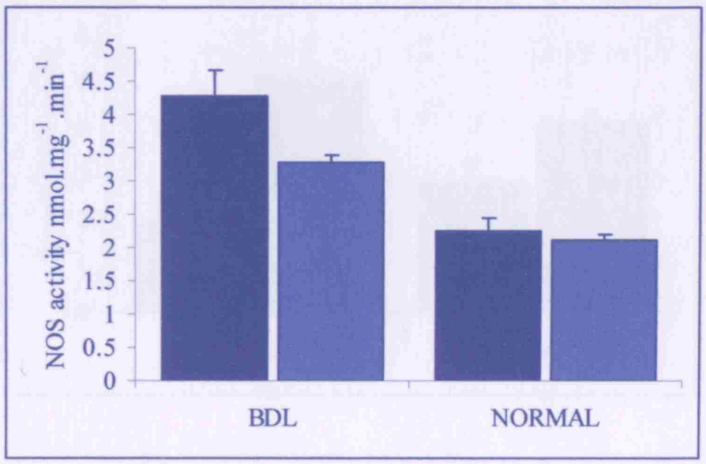


Figure 4.9 Hepatic NOS activity is increased in BDL cirrhotic rats. Chronic administration of lipoic acid significantly attenuated the increase in hepatic NOS activity, but had no effect on normal rats. Solid bars indicate rats which were not treated with lipoic acid. n = 6 in all groups.

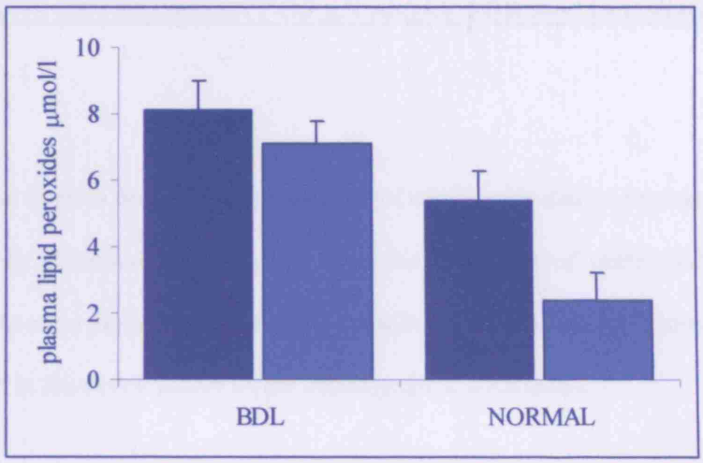


Figure 4.10 Lipid peroxide levels are significantly increased in BDL rats compared with normal controls. Levels in both groups were suppressed by lipoic acid, though this was only significant in the normal group. Solid bars indicate rats which were not treated with lipoic acid. n = 6 in all groups.

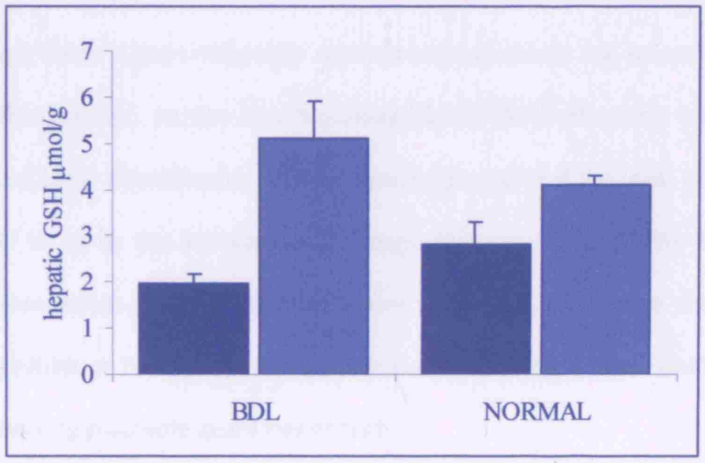


Figure 4.11 Hepatic reduced glutathione levels were lower in BDL rats when compared with normal controls, but this did not reach statistical significance. Lipoic acid treatment caused a rise in hepatic GSH in both groups, but only significantly in the BDL group. Solid bars indicate rats which were not treated with lipoic acid. n = 6 in all groups.

CHAPTER 5 - THE DEVELOPMENT OF AN ASSAY FOR S-NITROSTHIOLS

5.1 AIMS

In chronic liver disease there is both an overproduction of nitric oxide and an increased level of oxidant injury. It may therefore be predicted, from the chemistry of nitric oxide, that S-nitrosothiols are produced in excess. As these are vasodilator compounds with long half lives they may be important in the development of the hyperdynamic circulation.

A significant limitation in examining the role of these compounds in liver disease is the absence of an effective assay for them in plasma. A major component of the work presented in this thesis involves the development of an assay for plasma S-nitrosothiols.

At its most simple level the most important test for any assay in biological fluids, such as plasma, is whether one can reliably measure known amounts of authentic standards after they have been added to such fluids at physiologically relevant concentrations and recover the added amount in a reproducible manner. In this case S-nitrosoalbumin (SNO-albumin) was prepared and used as the standard in all experiments. Indirect studies predict that the level of plasma S-nitrosothiols are likely to be in the low nanomolar range (*Naseem et al. 1996*). In order to measure such low concentrations a highly sensitive sensor is required. The assay was therefore directed at measuring nitrite or NO release from S-nitrosothiols, using a nitric oxide analyser which is capable of detecting picomole quantities of both.

The overall aim was therefore to detect SNO-albumin added to plasma in a stoichiometric manner, and furthermore to produce a standard curve that was parallel to one obtained in a buffer, as illustrated in figure 5.1

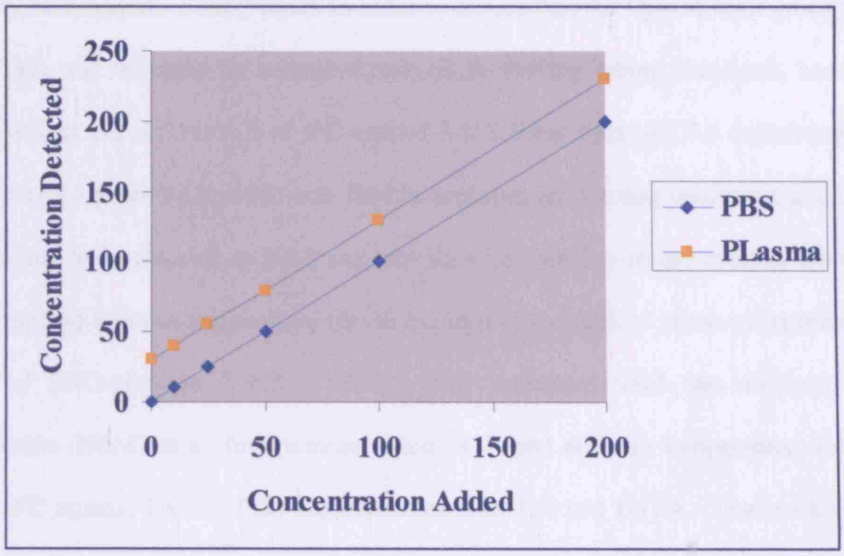


Figure 5.1 An ideal standard curve for the detection of SNO-albumin in plasma and phosphate buffered saline, where the Y axis intersect in plasma is equivalent to baseline concentration.

With the development of an assay it would be possible to quantitate the level of circulating S-nitrosothiols and make a comparison with animal models of and patients with liver disease.

5.2 METHODS

5.2.1 Chemicals

All chemicals used in the development of the assay were purchased from Sigma (Poole, Dorset, UK).

5.2.2 Preparation of S-nitrosoalbumin

S-nitrosoalbumin was prepared by transnitrosating albumin with another S-nitrosothiol, in this case S-nitrosocysteine. All of the steps were carried out in the dark and in the presence of diethylenetriamine pentaacetic acid (DTPA), as both ultraviolet light and divalent metal ions lead to decomposition of S-nitrosothiols. Human serum albumin (20 mg/ml) was treated with 2 mM dithiothreitol in phosphate buffered saline (PBS), together with 100 μM DTPA and gently

stirred at room temperature for 2 hours in order to ensure that the Cys-34 thiol group was fully reduced. This was followed by extensive dialysis in Visking tubing (Medicell, London, UK; molecular weight cut off 14,000) at 4°C against 3 x 3 litres PBS, pH 7.4 containing 100 μM DTPA. S-nitrosocysteine (10 mM) was freshly prepared by reacting equimolar sodium nitrite with L-cysteine hydrochloride at pH 2 and was then incubated with the reduced albumin (150 μM or 10 mg/ml) at room temperature for 30 minutes in the dark to allow transnitrosation and formation of SNO-albumin (yield > 95%). Any unreacted thiol was alkylated with N-ethylmaleimide (NEM) at a final concentration of 1 mM at room temperature, followed by dialysis at 4°C against 3 x 3 L PBS supplemented with 100 μM DTPA. The stock solution of SNO-albumin (≈ 145 μM) was stored at –20°C and the concentration of SNO-albumin was determined immediately prior to use by measurement of mercury displaceable nitrite using the Saville reaction (*see below*).

5.2.3 Nitric Oxide and Nitrite Measurement

Nitric oxide was measured using the Sievers Instruments Model 280 Nitric Oxide Analyser (NOA™; Sievers, Boulder, USA) which is a high-sensitivity detector based on a gas-phase chemiluminescent reaction between nitric oxide and ozone:

$$NO + O_3 \rightarrow NO_2^* + O_2$$

$$NO_2^* \rightarrow NO_2 + h\nu$$

Nitric oxide and ozone are mixed in a small volume (≈ 20mL) reaction cell. This small volume permits measurement of low concentration of NO at low flow rates and produces sharp peaks for analysis of liquid samples. Emission from electronically excited nitrogen dioxide is in the red and near-infrared region of the spectrum, and is detected by a red-sensitive photomultiplier tube, cooled to –10°C in order to give maximum sensitivity. The sensitivity for measurement of

NO and its reaction products in liquid samples is ≈ 1 picomole. Data collection and analysis is performed using NOAnalysis™ software (Sievers, Boulder, USA).

NO is formed in the reaction chamber, and is then purged using an inert gas, in this case nitrogen, and passages to the photomultiplier tube where it can be quantified as described above following its chemiluminescense reaction – *see figure 5.2*

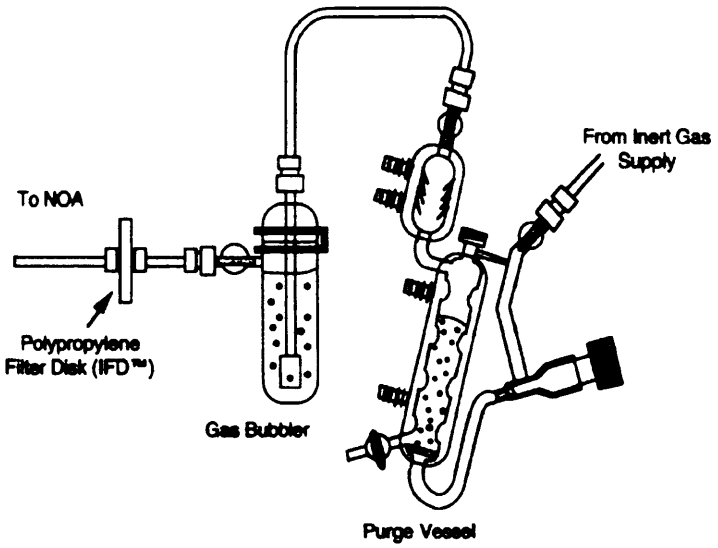


Figure 5.2 Nitric oxide analyser

In order to measure nitrite concentration the purge vessel contains a reducing agent (1% wt/vol of potassium iodide in acetic acid) to convert nitrite to nitric oxide.

$$I^- + NO_2^- + 2H^+ \rightarrow NO + \tfrac{1}{2} I_2 + H_2O$$

The analyser is mass sensitive and the response will depend upon the quantity rather than the concentration, such that 1µl of a 10µM standard will give the same response as injection of 100µl of a 0.1µM solution.

One of the main limitations of this system described is in the measurement of biological samples containing proteins, as the constant purging leads to significant foaming. An antifoaming agent can be added to the purge vessel, with some success, to minimise foaming from samples that contain protein.

In all experiments described, prior to injection of samples, a standard curve of nitrite concentrations between a range of 1nM and 1μM, was performed in order to calibrate the analyser.

5.2.4 The Saville Reaction

The stock solution of SNO-albumin was quantified by the method of Saville (*1958*). In brief, 20μl of sample is acidified by adding 200μl of 0.5M hydrochloric acid. After 2 minutes, 200μl of ammonium sulfamate is added, to give a final concentration of 0.5%, in order to remove background nitrite. The mixture is then supplemented with mercuric chloride, final concentration 0.1% and sulfanilamide at a final concentration of 0.5%

The nitrosonium ions released by mercuric ions react rapidly with aromatic amines, such as sulfanilamide, which then reacts with the Griess reagent (N-1-napthylethylenediamine dihydrochloride) to form an intensely coloured azo dye that can be quantitated spectrophotometrically, by scanning at a wavelength of 540nm, using an extinction co-efficient of $50{,}000M^{-1}cm^{-1}$. The method was confirmed against a standard curve using a known concentration of SNO-glutathione.

5.2.5 Plasma Thiol Measurement

Measurement of free thiol groups was performed using a spectrophotometric assay based on 2,2-dithiobisnitrobenzoic acid (DTNB or Ellman's reagent). In brief, 200μl aliquots of plasma are added to Tris buffer pH 8.2, together with Ellmans reagent and methanol to bring a final volume of 4ml . The colour is allowed to develop for 10-15 minutes, samples are then

centrifuged at 3,000g and absorbance of the supernatant is measured at 412nm. Total free thiol groups are measured using a coefficient of extinction of 13,600 $cm^{-1}M^{-1}$.

5.3 RESULTS

5.3.1 STEP 1 – Modification of the Saville Method

The Saville method for the quantitation of S-nitrosothiols relies upon mercuric liberation of nitrite followed by rapid reaction with sulfanilamide and then the Griess reagent. The sensitivity of the nitric oxide analyser is significantly greater than spectrophotometric detection of the azo dye formed with the Griess reagent, being sufficient to measure 1 picomole of NO, equivalent to 100µl of a 10 nanomolar solution.

The first and most simple approach to developing an assay for S-nitrosothiols was therefore to employ a more sensitive detector to look at mercuric generation of nitrite. Neither ammonium sulfamate or sulfanilamide were used in this series of experiments, as they would react with the newly generated nitrite, therefore the background concentration of nitrite had to be subtracted from the total, as illustrated in figure 5.3.

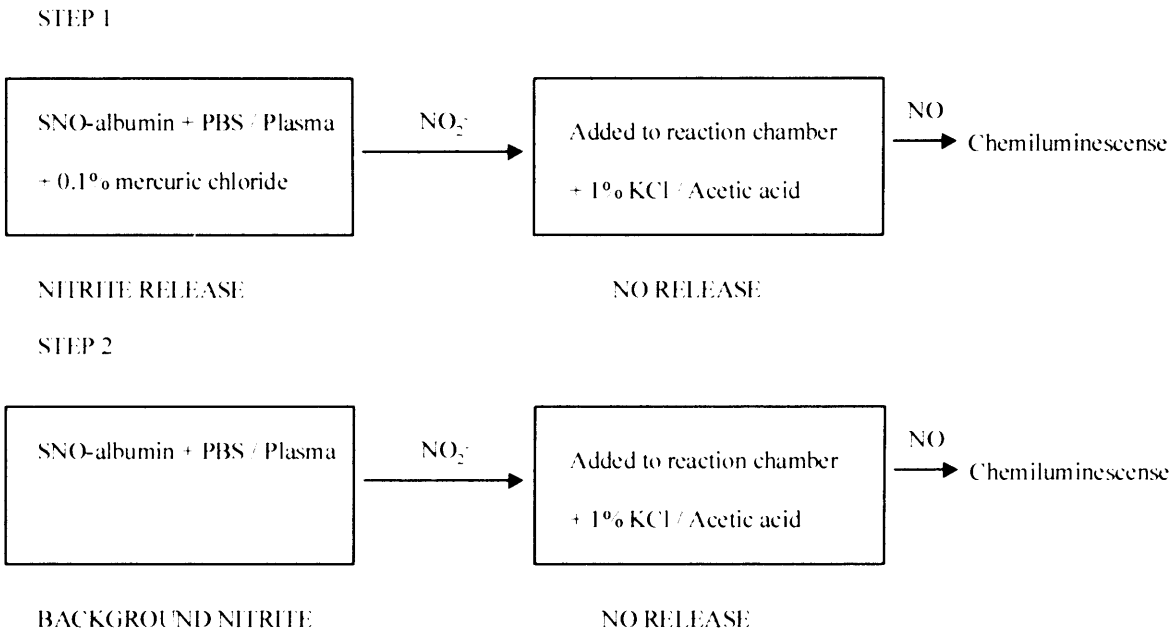


Figure 5.3 Modified Saville reaction. Estimated RSNO concentration is equivalent to the NO measured in step 2 subtracted from step 1

This method was successful at detecting SNO-albumin diluted in PBS, down to concentrations as low as 100nM. In plasma the level of background nitrite was so high (>300 nM) that the small incremental differences following addition of 1% mercuric chloride at the lower end of the dose response curve could not be accurately reproduced or quantitated.

5.3.2 STEP 2 – Transnitrosation and Metal Cation Mediated NO Release

The second approach to quantitate S-nitrosothiols was based upon the divalent cation liberation of nitric oxide from low molecular weight nitrosothiols. S-Nitrosoalbumin was diluted in 0.5M PBS in the reaction chamber of the nitric oxide analyser. An excess of low molecular weight thiol, either cysteine or N-acetyl cysteine (final concentration 1 mM) was then added to the reaction chamber for 5 minutes, in order to transnitrosate from high molecular weight to low molecular weight S-nitrosothiols. This step was then followed by addition of varying concentrations of either copper sulphate or mercuric chloride again directly into the reaction chamber. Nitric oxide release was then quantitated by chemiluminescense, as shown in figure 5.4, and then plotted against concentrations of a standard curve of sodium nitrite.

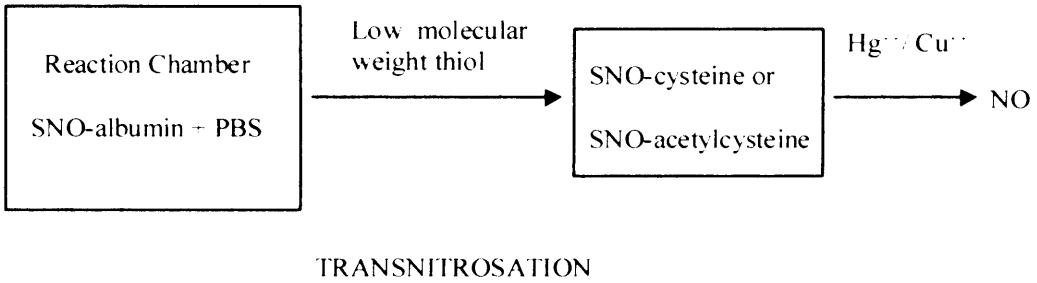


Figure 5.4 Transnitrosation followed by divalent cation release of NO

Although reasonable standard curves were obtained, this approach was limited by three factors. The addition of progressively increasing quantities of albumin to the reaction chamber caused marked foaming due to the continuous sparging of the reaction mixture with nitrogen. Second, experiments adding exogenous sodium nitrite to the standard solutions demonstrated that the reaction conditions employed lead to some NO release from nitrite itself. Thirdly the rate of NO release, although stoichiometric within samples, varied between samples. Clearly this would have significant implications when trying to quantitate S-nitrosothiols in biological samples such as plasma. This approach was therefore abandoned, and because of the problems of background nitrite, methods to eliminate nitrite were explored.

5.3.3 STEP 3 – Elimination of Background Nitrite

Having encountered significant problems with methodology due to the presence of nitrite ions in biological samples, the two chemical approaches described in the original Saville method for removing background nitrite were compared, namely addition of ammonium sulfamate or sulfanilamide, both under acidic conditions.

1) Reaction of nitrite with ammonium sulfamate (final concentration 0.5% in 0.045M hydrochloric acid)

$$H^+ + NO_2^- \rightarrow HONO$$

$$HONO + NH_4SO_3NH_2 \rightarrow NH_4^+HSO_4^- + N_2 + H_2O$$

2) Reaction of nitrite with the aromatic amine sulfanilamide (final concentration 0.5% in 0.1M hydrochloric acid)

$$H^+ + NO_2^- \rightarrow HONO$$

$$HONO + Ar\text{-}NH_2.HX \rightarrow [ArN{\equiv}N]^+X^- + 2H_20$$

Sodium nitrite was added to plasma at final concentrations of 1, 2.5 and 5μM. In parallel samples either ammonium sulphamate or sulfanilamide were added. Residual nitrite was then quantitated using the chemiluminescense assay, followed by comparison against a standard curve of sodium nitrite. As is shown in figure 5.5, at a final concentrations of 5μM acidified sulfanilamide was more effective than acidified ammonium sulfamate in removing exogenous nitrite.

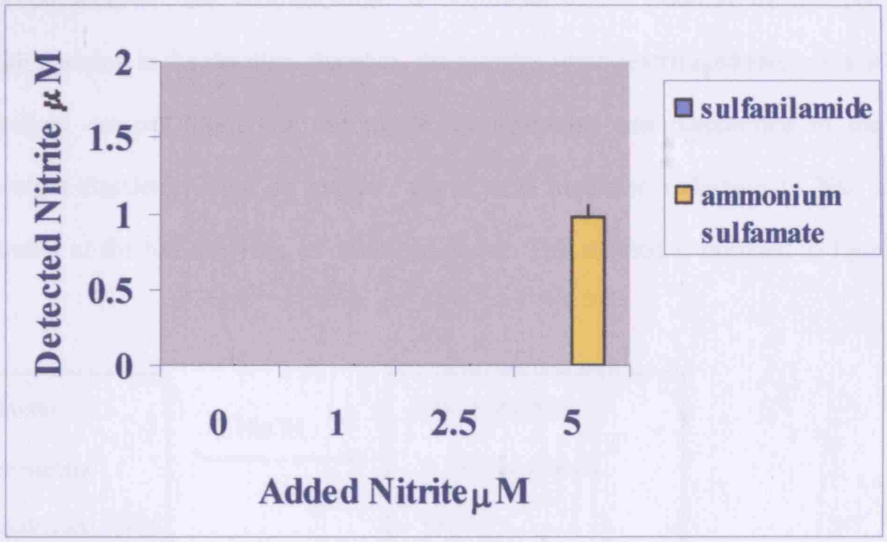


Figure 5.5 A comparison between ammonium sulphamate and sulfanilamide at removing nitrite ions added to plasma, demonstrating superior elimination with sulphanilamide

In order to ensure that this method of nitrite elimination did not lead to artefactual generation of S-nitrosothiols, albumin was incubated with acidified sulphanilamide and a 5μM sodium nitrite solution. The albumin was then separated by filtration through a 30, 000 molecular weight cut off filter and resuspended in PBS. Addition of mercuric chloride to the albumin did not lead to release of any nitrite, confirming that S-nitrosoalbumin is not generated by this method.

5.3.4 STEP 4 – Nitrite Elimination followed by Divalent Metal Cation or LMW Thiol Mediated NO Release

Once a successful method for elimination of background nitrite had been established, a further modification of the initial Saville assay was investigated. As the elimination of nitrite by sulfanilamide only occurs under acid conditions, and as S-nitrosothiols are relatively stable under acidic conditions, it was hypothesised that back titrating the pH of the solution to neutral pH prior to the addition of a divalent cation or low molecular weight thiol would lead to nitrite liberation proportional to the concentration of S-nitrosothiols. Because of the previous problems with foaming in the reaction chamber, the samples were centrifuged through a 30,000 molecular weight cut off filter, and the nitrite concentration was determined in the low molecular weight fraction., using an iodide / acetic acid mediated reduction to NO in the reaction chamber of the NO analyser, as described above. This method is outlined in figure 5.6 below.

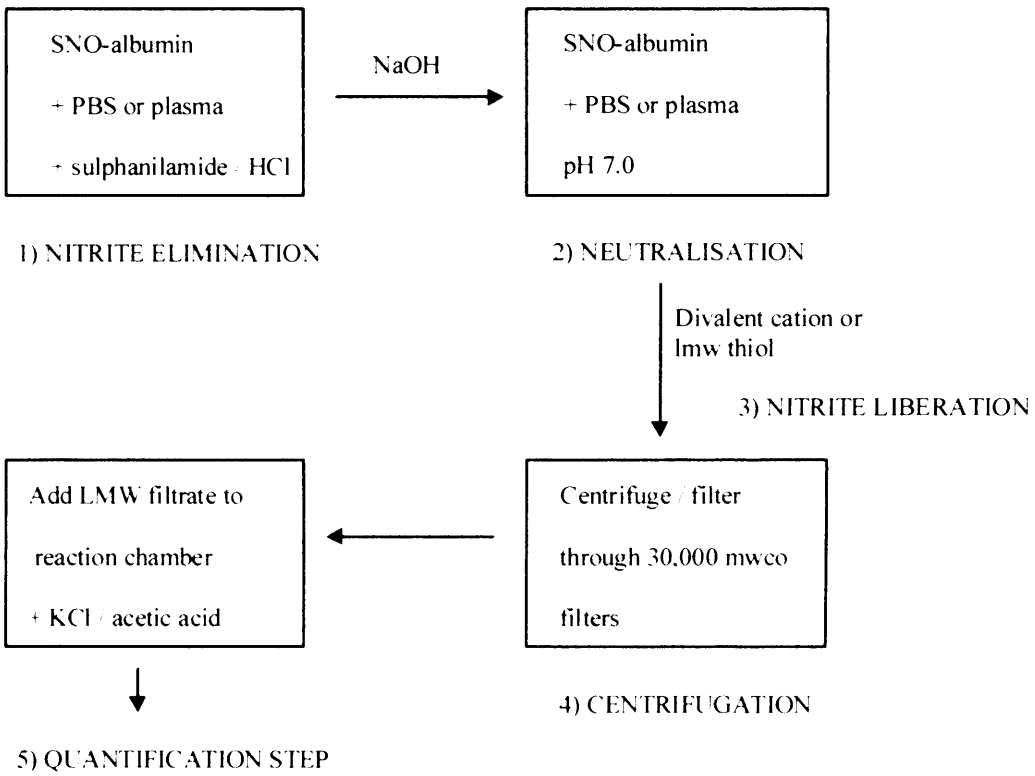


Figure 5.6 Nitrite elimination followed by pH neutralisation and nitrite liberation in order to quantify RSNOs.

This method had the advantage of not having to subtract high background nitrite levels from the total. In order to break the S-N bond and form nitrite, Cu^{++}, Hg^{++}, cysteine and N-acetylcysteine were tried in various concentrations and various combinations, to elucidate the most efficient concentrations. A final concentration of Hg^{++} 1mM or of cysteine 10mM were found to be most effective for SNO-albumin in PBS.

Standard curves were then obtained attempting to recover nitrite from SNO-albumin added to both PBS and plasma, with subsequent analysis by chemiluminescense as described above. The results are shown below in figure 5.7, with the signal obtained being compared with a standard curve obtained from sodium nitrite solution over a similar concentration range.

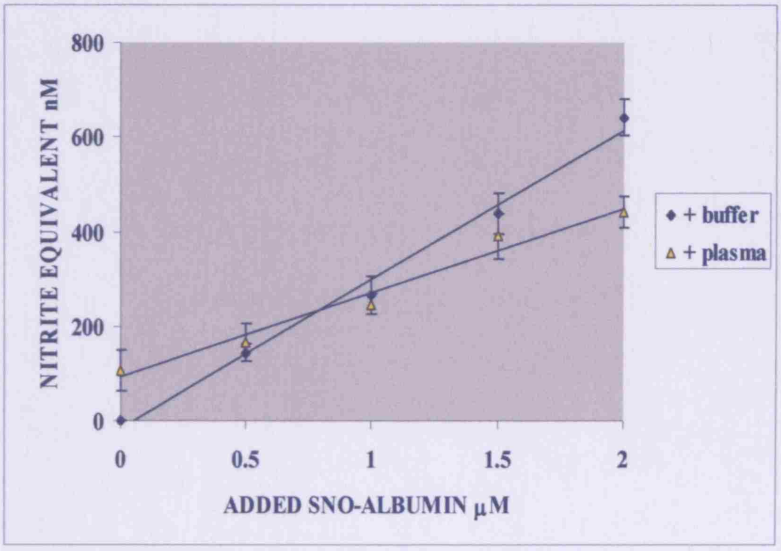


Figure 5.7 Application of step 4 to quantifying SNO-albumin added to both PBS and plasma (n=4). Values are expressed as mean ± s.e.m (n = 3)

As shown above linear standard curves were obtained in buffer, although only approximately 30% of the anticipated nitrite was recovered, based upon the calculated concentration of SNO-albumin, using the Saville method prior to serial dilutions. However, when SNO-albumin was

added to plasma, although there appeared to be endogenous S-nitrosothiols at a concentration of between 50 – 100nM, there was no stoichiometric recovery of added S-NO albumin.

5.3.5 STEP 5 - Direct Addition of Samples to the Reaction Chamber

In spite of the effective elimination of nitrite from biological samples, the previous method had failed to stoichiometrically release NO from SNO-albumin added to plasma. The next step was therefore to return to adding samples directly to the reaction chamber, following nitrite elimination with sulfanilamide. 100 µl samples were injected into the reaction chamber, which contained 10 ml PBS, together with a variety of catalysts including low molecular weight thiols and divalent metal ions.

Although all of the catalysts were able to effectively release detectable NO from added SNO-albumin, the anticipated signals were only 10% of those predicted from standard curves of equimolar concentrations of sodium nitrite, leading to a significant negative impact on the sensitivity of the assay (*results not shown*). The reasons for this were not clear, but may reflect the effect of the pH on NO release. As the reaction chamber contained a physiological buffer solution, it may have been that nitrite was preferentially generated by the breakdown of SNO-albumin rather than NO.

If nitrite was being generated this could then be converted to NO quite simply by adding glacial acetic acid and potassium iodide to the reaction chamber rather than PBS. Any sulfanilamide in the sample would be extensively diluted by the large volumes employed. In order to release NO from SNO-albumin the same catalysts were used, though previous results showed a greater efficiency with divalent metal ions than low molecular weight thiols. The solution was constantly purged with nitrogen and NO release was detected by chemiluminescense as described above. (*see figure 5.8*).

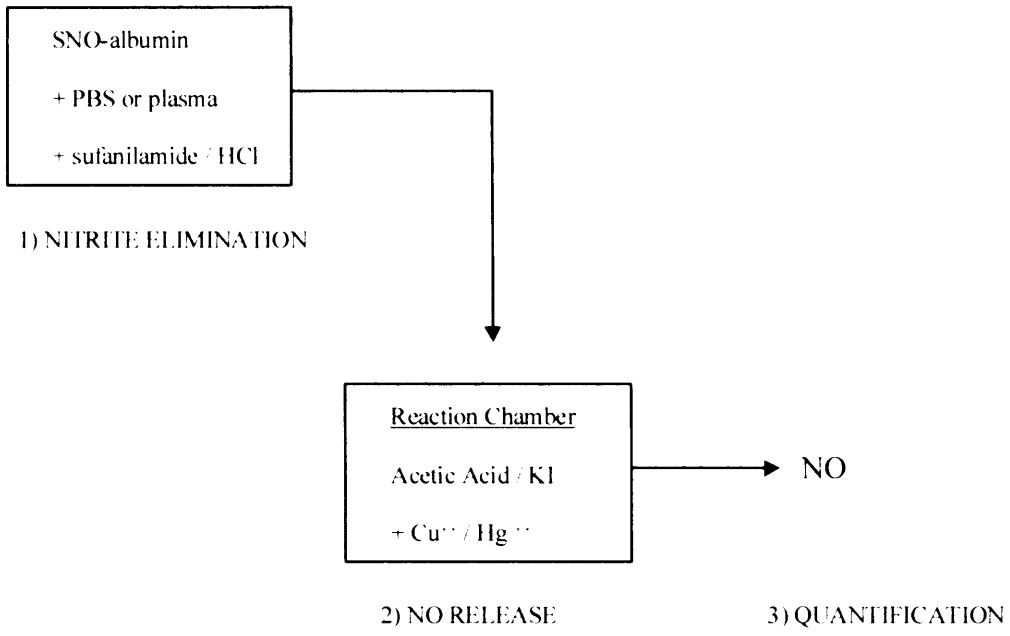


Figure 5.8 Step 5. Nitrite elimination followed by NO release in the reaction chamber.

Once the background readings had settled either mercuric chloride or copper sulphate were added to the reaction chamber at varying concentrations. Addition of copper sulphate lead to the reactant turning brown immediately, which is characteristic of aqueous solutions of I_3^-. 100µl aliquots of 1µM SNO-albumin were then added over a final mercuric(II) or copper(II) concentration range of 0.1 to 10mM and the NO release was quantified. Copper sulphate was superior to mercuric chloride and at a final concentration of 5mM gave an NO signal of between 90 and 95% of that obtained from 100µl of 1µM sodium nitrite, as measured by acetic acid/potassium iodide alone. Although the amount of NO recovered was less than predicted from standard curves of sodium nitrite, the recovery was stoichiometric, however, the lowest concentration that could be accurately and reproducibly detected in plasma was 200 nM.

Once this method had been developed all subsequent modifications were employed to try and increase the sensitivity of the assay in order to detect S-nitrosothiols at concentrations in the low nanomolar range, as these were the predicted levels for healthy human plasma.

5.3.6 STEP 6 – The Sample Volume

The major limiting factor to the sensitivity of the assay was the volume of sample which could be added to the reaction chamber. As the analyser is mass sensitive rather than concentration sensitive the main direction to increase sensitivity was to increase the volume of sample that could be analysed.

In order to detect NO release within the reaction chamber, the reactant is constantly sparged with nitrogen. Due to the presence of plasma proteins, this has the effect of causing foaming and potential blockage of the outlet tubes, with subsequent reduction in the sharpness of the peak and difficulty in their integration. The maximum volume of plasma that could be added to the reaction chamber was 100µl, and even then the reaction mixture had to be changed after each sample was added.

In order to reduce the problem with foaming, protein precipitation was not feasible, as the majority of circulating S-nitrosothiols are likely to exist as S-nitrosoalbumin. Addition of antifoam™ was, in part, effective but only with sample volumes of up to 200µl.

To increase the sensitivity of the assay larger sample volumes were required. A new reaction chamber was specially commissioned increasing the reaction cell volume from 20 to 50ml. With addition of antifoam™ to samples in a ratio of 1:9, and cleaning out the reaction chamber following each sample assay, this allowed the sample volume to be increased from 100µl to 2ml, with a potential 20 fold increase in the sensitivity of the assay.

To ensure that sample volume did not effect the reproducibility of the assay a simple experiment was performed to demonstrate that different volumes of a plasma sample with SNO-albumin, added to give a final concentration of 1μM, gave a linear response. *See figure 5.9*

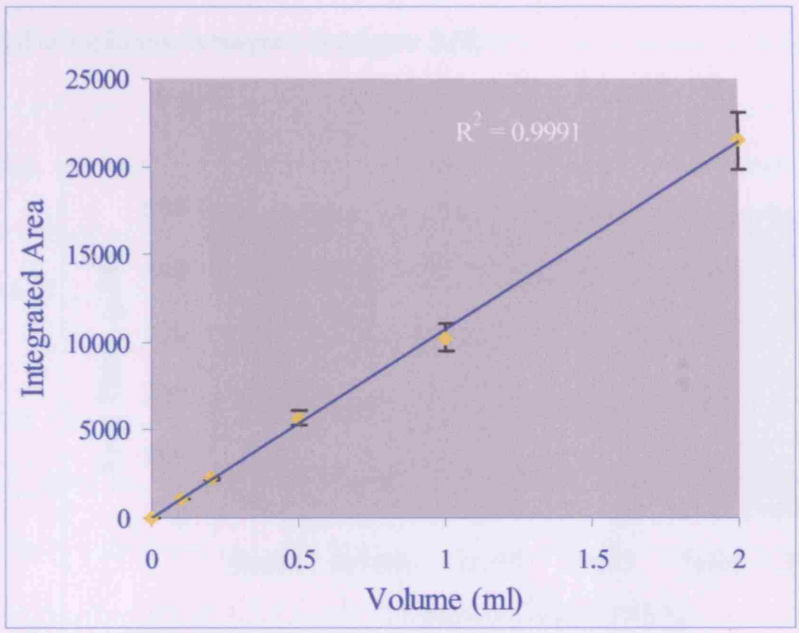


Figure 5.9 Increasing the volume of the sample added to the reaction chamber had no effect on NO release and detection (n = 4)

With this modification to the reaction chamber there was a reduction in the sharpness of the peaks for the detection of nitric oxide release. Nevertheless there was a 10 fold increase in sensitivity of the assay, with a lower limit of 20 nM.

5.3.7 STEP 7- Alkylation of Free Thiol Groups

In plasma the free thiol concentration is ~ 500μM/l. The major source is albumin (>90%) with lesser concentrations from cysteine and glutathione (*Brigham et al. 1960, Hagenfeldt et al. 1978*). Low molecular weight thiols lead to degradation of S-nitrosothiols by way of transnitrosation reactions. It was hypothesised that the presence of free thiols in plasma during the assay process might lead to degradation of RSNOs before they were quantified. Therefore in

order to stabilise S-nitrosothiols free thiol groups were alkylated using N-ethylmaleimide to prevent transnitrosation.

To determine the effective concentration of NEM required to fully block all free thiols, plasma was incubated with varying concentrations of NEM for 15 minutes, following which free thiols were assayed using Ellman's reagent. *See figure 5.10.*

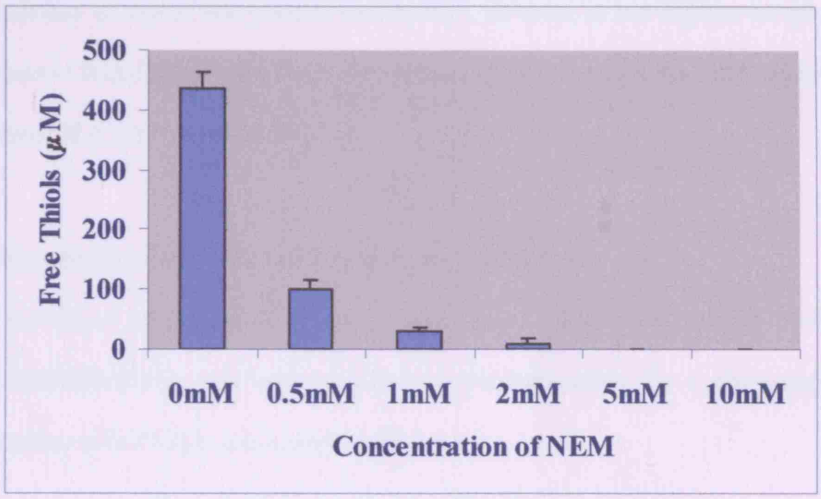


Figure 5.10 Following addition of the alkylating agent NEM to plasma free thiols were still detected at a final concentration of NEM of 2mM but not at 5mM (n = 4)

The lower than expected concentration of free thiols in the control plasma (436 ± 26μM) may reflect oxidation of the plasma during the 15 minute incubation period prior to addition of Ellmans reagent.

Following this all subsequent plasma samples were treated with NEM at a final concentration of 5 mM prior to assaying for S-nitrosothiols

5.3.8 STEP 8 - Effect of Temperature

Once the assay had been developed, in order to increase its sensitivity further, the effect of the temperature of the reaction was examined. Using a water bath the temperature of the water jacket around the reaction chamber was varied. Water was circulated at room temperature, 30°C, 50°C, 70°C and 90°C. A single sample of plasma with a final concentration of SNO-albumin of 1μM was assayed at these different temperatures. With increasing temperature there was a modest increase in signal size (results not shown), however at the highest temperature there was an excess of foaming and blockage. For subsequent experiments the water jacket was therefore maintained at a temperature of 70°C.

5.3.9 STEP 9 - Standard curve of SNO-albumin Added to Plasma

As a result of the above modifications of the RSNO assay, described in section 5.3.5, the following final methododology was used to obtain a standard curve for a physiologically relevant concentration of SNO-albumin added to plasma.

5.3.9.i Sample Preparation

The final sample volume was 2 ml, and was made up of the following components

i) Plasma – 1700 μl

ii) 200mM N-ethylmaleimide – 50 μl (final concentration 5 mM)

iii) SNO-albumin – 50 μl (diluted to give final concentrations of 5, 10, 25, 50, 75 and 100 nM)

iv) Antifoam™ (Sievers, Boulder, USA) – (diluted 1:30) – 200 μl

5.3.9.ii Nitric Oxide Analyser Preparation

As described above, the reaction chamber was custom designed to accommodate larger sample volumes but was otherwise identical in design to a commercially available glass reaction chamber (Sievers Radical Purger™). It contained 8 ml glacial acetic acid and 2 ml potassium

iodide (50 mg/ml) and was kept at 70°C via a water jacket. One minute prior to injection of the sample 200 µl of copper (II) sulphate (200 mM) was added. This solution was constantly purged with nitrogen, and used only once per sample. The outlet of the gas stream was directly passed into a Sievers Instruments Model 280 Nitric Oxide Analyser (NOA™; Sievers, Boulder, USA). Data collection and analysis was performed using the NOAnalysis™ software.

5.3.9.iii Standard Curve

Once a stable background reading had been obtained samples were injected rapidly into the reaction chamber by means of a Hamilton syringe and the release of NO was quantified by its chemiluminescent reaction with ozone.

Comparison was made of the NO signal recovered using a standard curve derived from an equimolar concentration range of SNO-albumin in phosphate buffered saline.

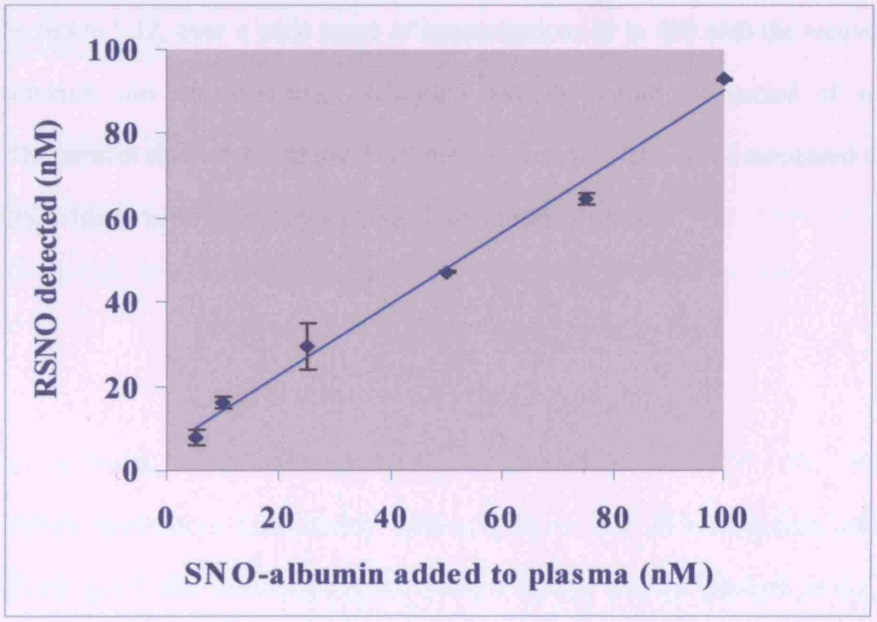


Figure 5.11 Final assay method for S-nitrosothiols in plasma, demonstrating stoichiometric recovery of SNO-albumin added to plasma. The recovery was approximately 95% of the signal predicted from detection of SNO-albumin in PBS. The lower limit of detection was 5 nM (n = 4). (*Marley et al 2000*)

5.3.10 STEP 10 - Assay of SNO-albumin Added to Whole Blood

The final step in the assay development was to determine whether significant degradation of SNO-albumin occurred during the centrifugation period necessary to obtain plasma from whole blood. Fresh blood was collected from the antecubital vein of human volunteers into a tube containing EDTA (final concentration 2 mM) and NEM (final concentration 5 mM), to chelate divalent metal ions and alkylate free thiol groups respectively, and was immediately placed on ice and covered with aluminium foil to prevent light degradation. Samples were then spun down in a pre-chilled (4°C) centrifuge at 2,500g for 5 minutes. To determine whether there was significant RSNO degradation during this sample preparation period, the recovery of known amounts of SNO-albumin added to whole blood was determined. The partitioning of SNO-albumin into the plasma phase was determined by correction using the haematocrit of the samples.

As is shown in figure 5.12, over a wide range of concentrations (0 to 400 nM) the recovery of added SNO-albumin was stoichiometric, indicating stability during the period of sample preparation. The parallel shift of the standard calibration curve in whole blood compared to that obtained in PBS is likely to be caused by endogenously present RSNO.

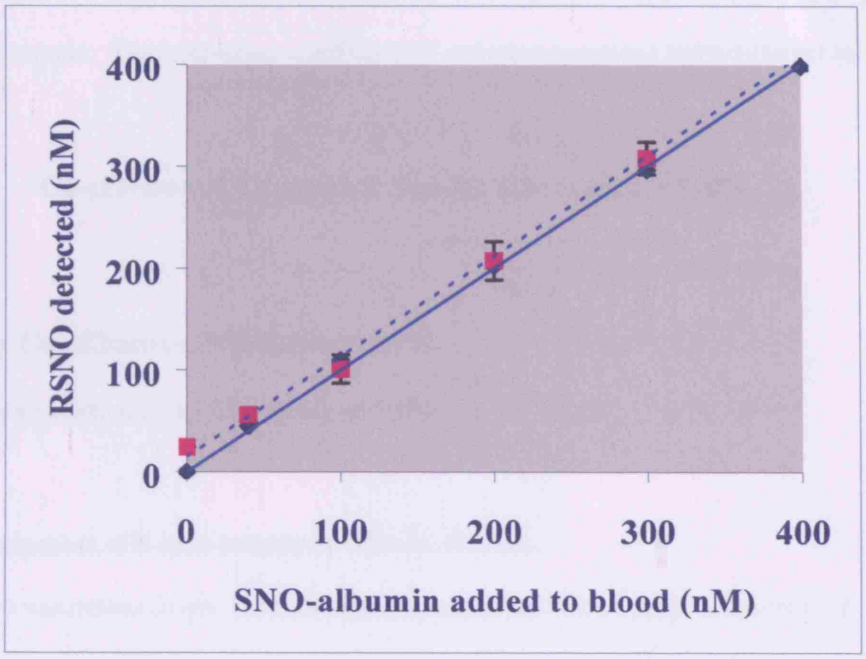


Figure 5.12 The recovery of SNO-albumin added to blood (dashed line) compared to that recovered from PBS (continuous line) (n = 4).

5.3.11 Determination of Reproducibility of the Assay

Having established that NO release could be detected stoichiometrically over a concentration range for SNO-albumin the intra-assay and inter-assay coefficient of variations were determined.

SNO-albumin was added to plasma to give a final concentration of 25, 50, 100, 500 and 1000nM. Standard curves were then obtained following assays of 8 different plasma samples. On one sample the assay was repeated 8 times. Using a best-fit line the gradient of the lines were calculated.

The intra-assay coefficient of variation was determined from the 8 repeated gradients obtained from a single sample. The inter-assay coefficient of variation compared the 8 different samples.

$$\textbf{Co-efficient of Variation} = \frac{\textbf{Standard Deviation x 100\%}}{\textbf{Mean}}$$

Intra-Assay Co-efficient of Variation = 3.7%

Inter-Assay Co-efficient of Variation = 4.3%

5.3.12 Measurement of S-nitrosothiols in Human Plasma

Venous blood was collected into prechilled tubes containing EDTA (final concentration 2 mM) and NEM (final concentration 5 mM), and centrifuged for 10 minutes at 1300g and 4°C. To obtain the low molecular weight fraction plasma was centrifuged through a 30,000 molecular weight cut off filter for 30 minutes at 13,000g.

Human plasma was obtained from 10 healthy volunteers. RSNOs were detectable in all subjects with a mean concentration of 28 ± 7 nM (n = 10). There was no detectable signal from the low molecular weight filtrate in any of the samples analysed, suggesting that the major forms of circulating RSNOs have a molecular weight of greatery than 30,000.

5.3.13 Measurement of S-nitrosothiols in Acute Bile Duct Ligated Rat Plasma

Plasma nitrosothiols were measured in rats that had undergone acute bile duct liagation, being studied at 48hours, and were compared with sham operated animals. This model is associated with acute liver injury (*Holt 2000*) . This showed that BDL increased s-nitrosothiol generation. Sham nitrosothiol levels were 20.1 ± 1.1 nM (n=4) and these levels increased to 36.8 ± 3.0 nM post BDL (n=4). When the two groups were compared using a student t-test there was a significant difference between the two groups, $P < 0.05$. *See figure 5.12*

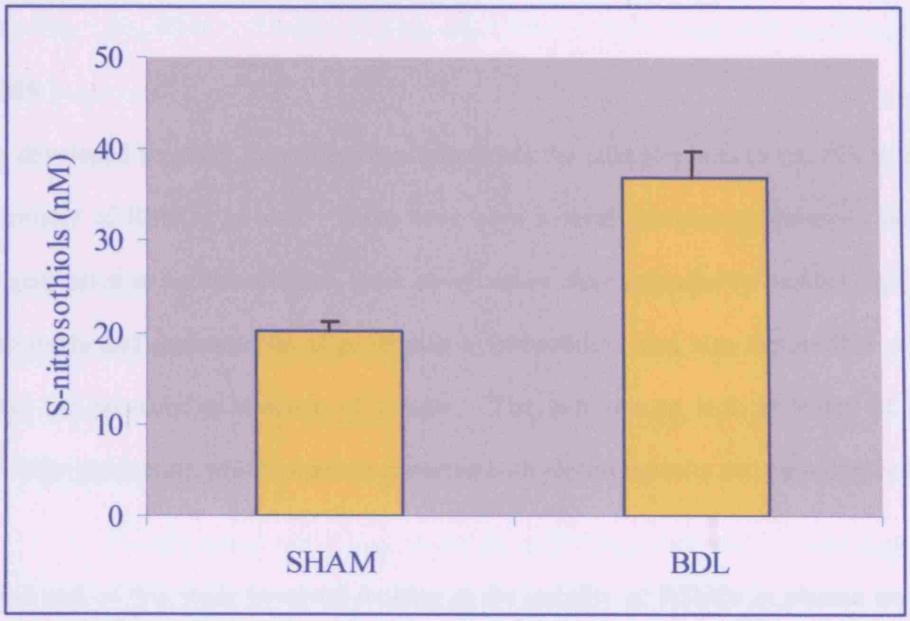


Figure 5.13 Plasma S-nitrosothiol levels are significantly increased by acute bile duct ligation ($P < 0.05$, $n = 4$)

CHAPTER 6 - THE BIOCHEMISTRY OF S-NITROSOTHIOLS *IN VIVO*

6.1 AIMS

Having developed an assay for plasma S-nitrosothiols the next step was to use this to examine the chemistry of RSNOs *in vivo*. There have been several previous publications looking at RSNO generation in buffer solutions, these observations were extended by looking at the ability of nitric oxide and peroxynitrite at generating s-nitrosothiols, and also factors that effect this including the presence or absence of oxygen. The aim was to look at levels of NO and peroxynitrite production which could be expected both physiologically and pathologically.

A second part of this study involved looking at the stability of RSNOs in plasma and factors whhich influenced this, in order to determine the likely steady state between production and destruction, and also to examine possible pharmacological means of manipulating them *in vivo*.

6.2 METHODS

6.2.1 Chemicals

S-nitrosoglutathione. SIN-1 (3-morpholino-sydnonimine), papaNONOate and detaNONOate were purchased from Cayman Chemicals Company (Ann Arbor, MI, USA). Xanthine oxidase and superoxide dismutase was purchased from Boehringer (Ingelheim, Germany). All other chemicals were from Sigma (Poole, UK).

6.2.1 Plasma Samples

Venous blood was collected into tubes containing EDTA (final concentration 2 mM), centrifuged for 10 minutes at 1300 x *g* and the plasma was used on the day of collection.

6.2.1 Measurement of S-nitrosothiols

RSNOs were quantified using the assay described in the previous chapter. For the purposes of this study low molecular weight S-nitrosothiols are those not retained by dialysis tubing (i.e.

Mwt < 14,000) or following centrifugation through an Ultrafree centrifugal tube (Millipore, Milford, MA, USA: Mwt < 30,000). Unless stated, three subjects were used in all experiments, and all data represents the mean ± SEM. NEM was used at a final concentration of 5 mM in all experiments when it was necessary to stabilise the RSNOs prior to measurement

6.3 THE GENERATION OF S-NITROSOTHIOLS IN PLASMA

6.3.1 Incubation of plasma with nitric oxide and nitric oxide donors

Plasma was incubated with three separate compounds to determine the effect of NO release on RSNO generation in plasma

i) A short acting nitric oxide donor papaNONOate was used to confirm generation

ii) Authentic nitric oxide was used to determine whether high or low molecular weight RSNOs were generated

iii) A long acting nitric oxide donor detaNONOate was used to look at the kinetics of RSNO formation

6.3.1.i PapaNONOate

Experiment: To determine whether NO was capable of generating S-nitrosothiols in plasma, plasma was incubated with a short half life NO donor, papaNONOate. Freshly obtained plasma (n = 3) was incubated with papaNONOate, at a final concentration of 100μM, and a final volume of 1ml, for a period of 15 minutes at 37°C in the dark. Parallel incubations were carried out with plasma pre-treated with the thiol blocking agent NEM, final concentration 5mM, in order to confirm specificity of RSNO formation. Following the incubation NEM was added to the plasma which had not previously been exposed to a thiol blocking agent, in order to stabilise any RSNOs formed. Before assaying RSNO concentrations in the treated plasma, any remaining papaNONOate in the sample was removed by extensive dialysis against 3 x 3l PBS containing 100μM DTPA and using Visking tubing with a molecular weight cut off of approximately 14,000 Da. Nitrite was removed by addition of sulphanilamide / HCl as described in the

previous chapter. To further confirm the specificity of RSNO generation a second series of incubations were carried out, but on this occasion plasma was subsequently treated with 0.1% mercuric chloride for 5 minutes prior to the addition of sulphanilamide / HCl and injection into the reaction vessel.

Affinity chromatography was used in order to determine whether RSNOs generated by NO donors were predominantly SNO-albumin. A chromatography column was filled with degassed blue sepharose 6 fast flow (Amersham Pharmacia, St. Albans, Herts, UK) prior to addition of plasma preincubated with papaNONOate (100 μM) for 15 minutes. The column was washed with start buffer (0.02M phosphate, 0.15M NaCl) and the albumin was then eluted with an elution buffer (0.02M phosphate, 2M NaCl). The albumin concentration was determined in the eluent using Bradford reagent against a standard curve generated from known concentrations of albumin. The total RSNO concentration was measured in the eluent and expressed as a percentage of the total RSNO concentration in a parallel plasma sample.

Results: Addition of papaNONOate (final concentration 100μM) to fresh human plasma and incubation for 15 minutes at 37°C, followed by a dialysis step, lead to the generation of high molecular weight compounds (>14,000) which released NO equivalent to an SNO-albumin concentration of 4.2 ± 0.6μM. Parallel samples of plasma, in which thiol groups had been alkylated with NEM prior to the addition of the NO donor, almost completely prevented formation of RSNOs, whereas addition of 0.1% mercuric chloride to the nitrosated plasma decreased the signal for RSNOs by 96%, confirming the specificity of this assay for S-nitrosothiols. (*See figure 6.1*)

Albumin was confirmed to be the major site for S-nitrosation. It was found that 69 ± 7% of the total RSNO generated in plasma could be recovered from the column as S-nitrosoalbumin.

6.3.1.ii Authentic NO

Experiment:: In the above experiment plasma was dialysed following treatment with NO donors, to prevent residual papaNONOate from interfering with the assay. As a result of this any low molecular weight RSNOs (Mwt < 14,000) would be lost into the dialysate. To determine the proportion of RSNO formed in the low and high molecular weight fractions of plasma an aqueous solution of nitric oxide was added to plasma. This was prepared by bubbling NO gas (99.5%) pure, BOC, UK) through water previously deoxygenated with argon for 20 minutes. Trace amounts of higher nitrogen oxides were removed by passing the nitric oxide through a 10% potassium hydroxide solution immediately prior to dissolution in the aqueous solution. The saturated solution was stored in a glass container and sealed with a small headspace under argon and used within 2 hours.

The aqueous solution was vortex mixed with plasma to give a final concentration of 20, 40, and 80μM in a volume of 1ml and incubated in the dark at 37°C for 15 minutes. At the end of this time RSNOs were stabilised by the addition of NEM. Because NO is rapidly dispersed and converted to nitrite under these conditions, a dialysis step is unnecessary. As demonstrated in the previous chapter excess nitrite does not result in S-nitrosothiol formation during sample preparation or interfere with the analytical method for RSNO employed. Following incubation, aliquots of plasma were then centrifuged through a 30,000 molecular weight cut off filter. The low molecular weight fraction and whole plasma were assayed separately to give total and low molecular weight RSNO concentrations.

Results: As shown in figure 6.2 both low and high molecular weight compounds are S-nitrosated following incubation of plasma with aqueous NO. In plasma the concentration of high molecular weight thiols (almost exclusively albumin) is ≈ 450μM, and the concentration of low molecular weight reduced thiols is ≈10μM (*Jones et al. 2000*). Presumably, because the high molecular weight thiols constitute more than 95% of total plasma thiols, the absolute

concentrations of high molecular weight RSNOs generated were higher. However the percentage of total RSNO in the low molecular weight fraction (≈5%) suggests there may be preferential nitrosation of low molecular weight thiols, either due to a direct action of NO or by transnitrosation reactions.

In spite of the high concentrations of nitrite that would be generated as a result of incubating aqueous NO with plasma, the low concentration of NO release from assay of the low molecular weight filtrate confirms that the assay conditions are very effective in removing background nitrite.

6.3.1.iii DetaNONOate

Experiment: In order to examine the kinetics of S-nitrosothiol formation by nitric oxide donors the long half-life compound detaNONOate was used as an alternative to papaNONOate. The rate of nitric oxide release in plasma was determined by adding detaNONOate to 2mls plasma, the rate of NO release per minute was then determined by chemiluminescense, and extrapolated to the different final concentrations used.

Subsequent experiments looked at the time course of RSNO formation by adding detaNONOate to plasma samples (n=3) at a final concentration of 250μM and a final volume of 1ml, and stopping the reaction at 0, 15, 30, 60, and 120 minutes by the addition of NEM (5mM). Following extensive dialysis to remove any possible contamination form detaNONOate the samples were assayed for S-nitrosothiols. *See figure 6.3*

In order to look at the effect of different rates of nitric oxide release on the production of RSNOs the above experiment was repeated but at varied final concentrations of detaNONOate of 0, 100, 250, 500, and 1000μM. All incubations in this case were for 120 minutes and again were stopped by the addition of NEM and followed by extensive dialysis prior to being assayed.

Results: Using the nitric oxide analyser the rate of NO release from detaNONOate at a final concentration of 250 μM was calculated at a constant rate of 15 nM/sec over a period of greater than 2 hours. As shown in figure 6.3 incubation of detaNONOate at a final concentration of 250 μM lead to a linear rate of accumulation of RSNOs at a rate of 3.4 ± 0.2 nM/min. In the second series of experiments detaNONOate was used at a range of concentrations (0-1000 μM) which releases NO at a rate calculated to be 0 – 60 nM/sec. Increasing the concentration of detaNONOate resulted in a progressive increase in RSNO formation (*see figure 6.4*). This relationship was linear if plotted as a function of the square of the rate of NO formation as shown in figure 6.5. This result is consistent with a reaction of two NO molecules and oxygen to form a nitrosating agent such as N_2O_3.

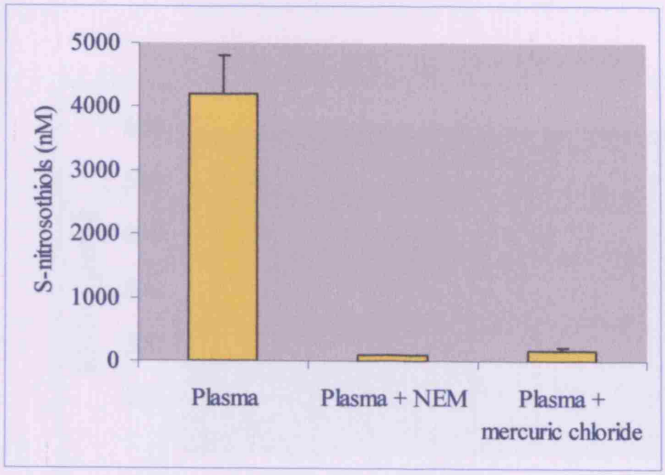


Figure 6.1 The generation of S-nitrosothiols by papaNONOate. PapaNONOate was added to plasma at a final concentration of 100 μM, and incubated for 15 minutes and then RSNOs were measured. Pretreatment with NEM blocked generation as did addition of mercuric chloride confirming they were S-nitrosothiols. (n = 3, all experiments). Data shown is mean ± s.e.m

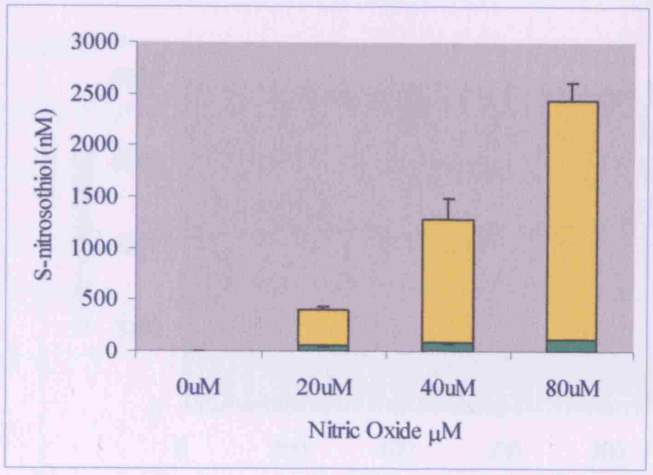


Figure 6.2 Formation of S-nitrosothiols in plasma by direct reaction with NO. Aqueous NO was added to plasma and total RSNO (yellow bar) and low molecular weight (green bar) RSNOs were measured. Despite large incresae in the concentrations of RSNOs in plasma, the concentration of low molecular weight RSNOs was less than 2% of the total (n = 3). Data shown is mean ± s.e.m

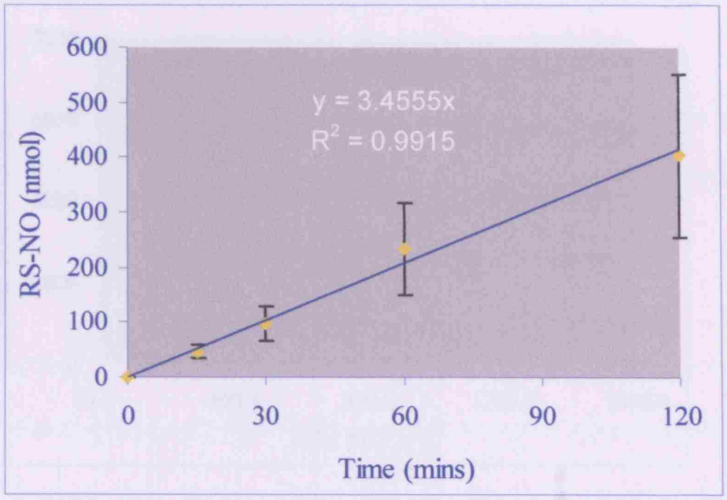


Figure 6.3 DetaNONOate (250 μM) was incubated with plasma for the times shown and the formation of RSNOs determined (n = 3). Data shown is mean ± s.e.m

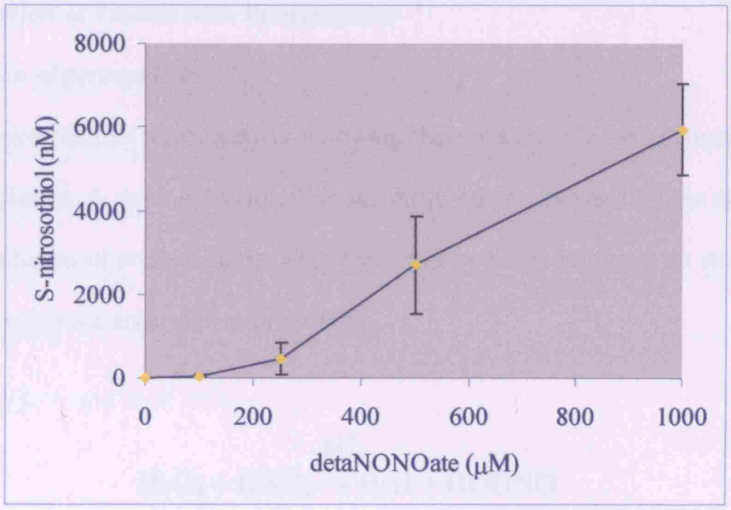


Figure 6.4 The effect of increasing the concentration of detaNONOate on the formation of RSNOs was determined at 2 hours (n = 3). Data shown is mean ± s.e.m

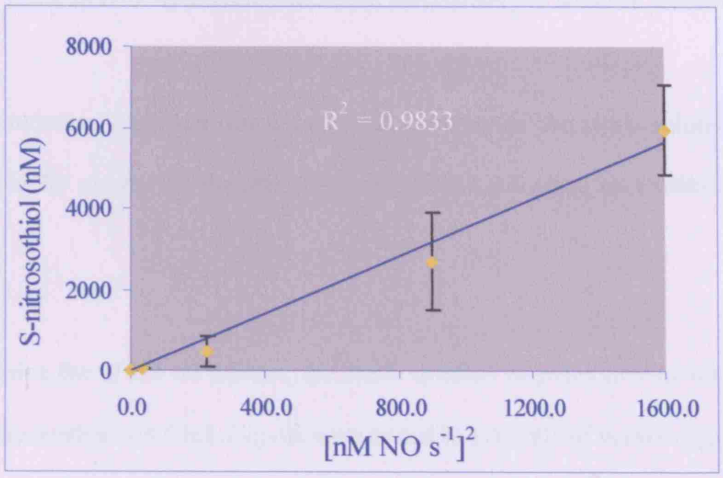


Figure 6.5 Reaction of detaNONOate with plasma with the RSNO concentration plotted against the square of the calculated NO flux. (n = 3). Data shown is mean ± s.e.m

6.3.2 The Incubation of Plasma with Peroxynitrite

6.3.2.i Preparation of peroxynitrite

S-nitrosothiol concentrations were assayed following the addition of varying concentrations of peroxynitrite to plasma. A stock solution of peroxynitrite was synthesised by the rapid reaction of an acidified solution of sodium nitrite with hydrogen peroxide followed by stabilisation of the product, peroxynitrous acid, with strong alkali.

$$H_2O_2 + HNO_2 \xrightarrow{H^+} H_2O + HOONO$$

$$HOONO + OH^- \leftrightarrow OONO^- + H_2O$$

In brief, an ice cold solution of 50ml 50mM sodium nitrite and 50ml 50mM hydrogen peroxide were stirred rapidly. 25ml of 1M hydrochloric acid was forced into the solution in a quench

flow reactor to form peroxynitrous acid, which even at 0°C has a half life of only a few seconds. Therefore 25ml 1.5M sodium hydroxide was added immediately to stabilise the product.

The final concentration was determined following dilution of the stock solution with 1.2M sodium hydroxide, by measuring the absorbance at 302nM and using an extinction coefficient of $1670M^{-1}$.

In order to examine the effect on plasma, the stock solution of peroxynitrite was diluted with 0.01 M sodium hydroxide and 50µl aliquots were added to 1.95 mls of vortexing plasma at 37°C to give final concentrations of 0, 10, 50, 100 and 500 µM, above this concentration the pH of the plasma was greater than 8.0. The samples were left for 15 minutes at 37°C in the dark prior to the addition of 5mM NEM they were then dialysed overnight and nitrite was removed by the addition of acidified sulphanilamide, as previously described, prior to assay.

6.3.2.ii Results

At concentrations above 10 µM the formation of high molecular weight RSNOs was proportional to the concentration of peroxynitrite (*see figure 6.6*). This relationship was not reflected at the lowest concentration, possibly as a result of competing reactions with other components found in plasma, such as tyrosine and methionine. Unlike the reaction with nitric oxide the kinetics for the reaction were first order. The conversion of peroxynitrite to S-nitrosothiol was approximately 0.25%.

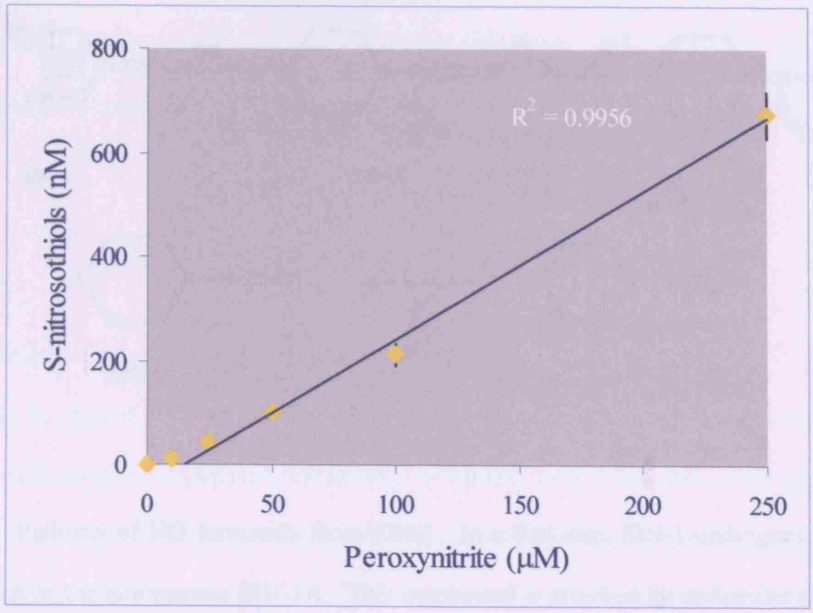


Figure 6.6 The generation of S-nitrosothiols following addition of peroxynitrite to plasma (n = 3). The error bars are too small to be seen accurately.

6.3.3 The Incubation of Plasma with Sydnonimine

6.3.3.i Methods

The sydnonimines are a class of heterocyclic NO donors, the best known compound in the class being SIN-1. They release equimolar quantities of superoxide radicals and NO, as shown in figure 6.7.

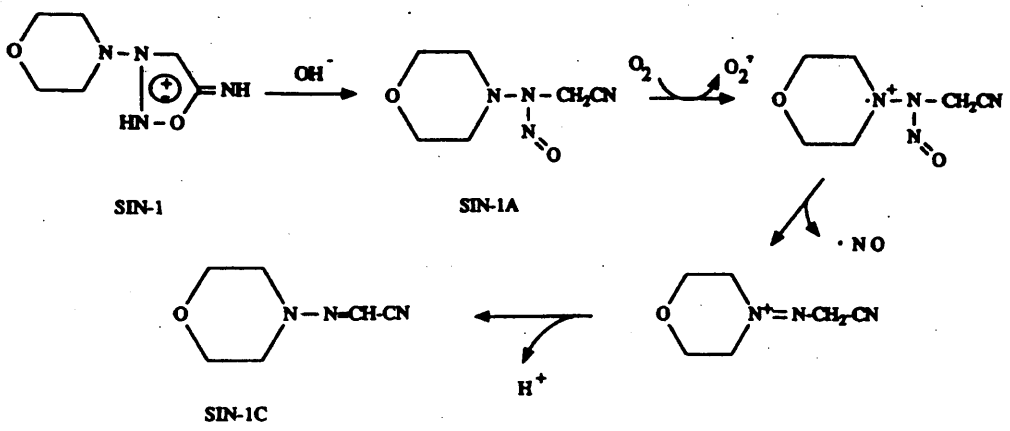


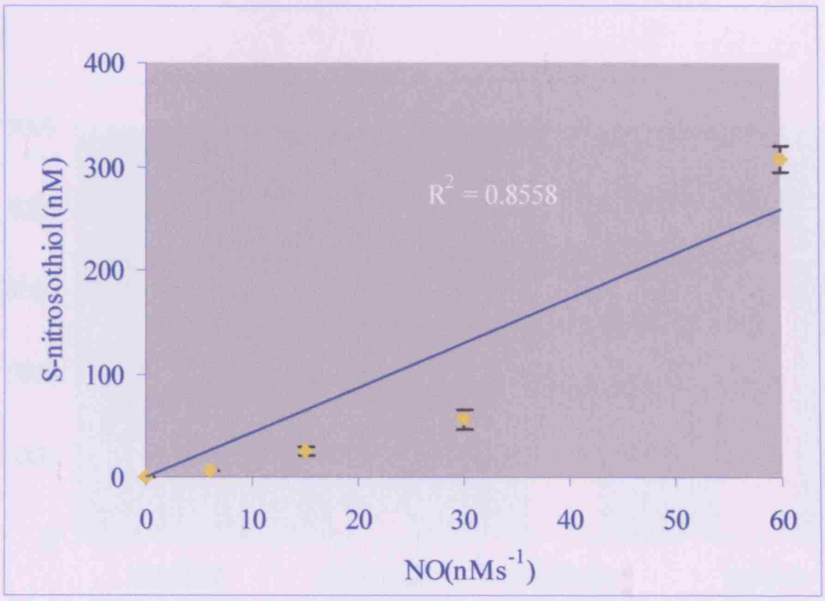
Figure 6.7 Pathway of NO formation from SIN-1. In a first step, SIN-1 undergoes hydrolytic ring opening to the nitrosamine SIN-1A. This compound is attacked by molecular oxygen and converted to a radical cation while oxygen is reduced to superoxide. The highly unstable radical compound is stabilised by NO split off and deprotonation (*from Feelisch 1991*)

SIN-1 was dissolved in deoxygenated PBS and left for 30 minutes to allow conversion to SIN-1A. This was then added to plasma to give a final concentrations of 0, 10, 20, 50, and 150 μM in a final volume of 1 ml. SIN-1A releases NO in a non-linear fashion with increasing concentrations (*Feelisch et al. 1989*), and these concentrations were calculated to give an NO flux of 0, 6, 15, 30, and 60 nM/sec i.e similar to those in the experiments using detaNONOate. Samples were incubated for 120 minutes and the reaction was stopped by the addition of NEM. Following a period of extensive dialysis, to remove any remaining SIN-1A, RSNO concentration was assayed.

To determine wehether superoxide inhibited the yield of S-nitrosothiols with SIN-1A, a further series of experiments was performed where SIN-1A (final concentration 50 μM) was incubated with plasma in the presence of varying concentrations of superoxide dismutase (0, 0.01, 0.1, and 1 kU/ml).

6.3.3.ii Results

As is shown in figure 6.8 increasing concentrations of SIN-1A and therefore increasing NO flux generated increasing concentrations of RSNOs in plasma over a two hour time period. The generation of RSNOs was only approximately 10% of the total concentration generated by equal NO fluxes using detaNONOate, presumably as a result of concurrent superoxide produxtion. Of interest the production of S-nitrosothiols appeared to follow second order reaction kinetics, as shown by a stronger correlation between RSNO concentration and the NO flux squared. Clearly concurrent superoxide genration effects the RSNO generation, as incubation with increasing concentrations of superoxide dismutase lead to significant increases in the final S-nitrosothiol concentration – *figure 6.9.*



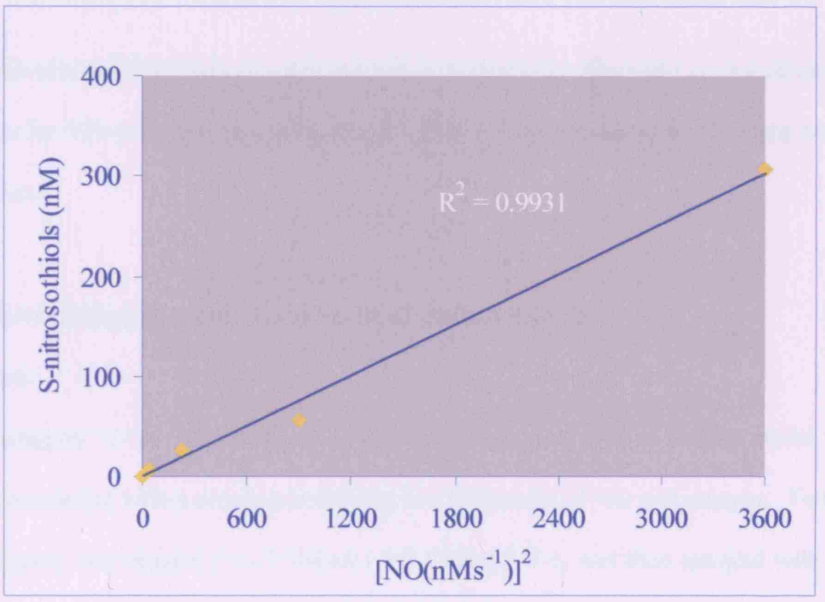


Figure 6.8 S-nitrosothiol generation following incubation of plasma with SIN-1A at varying concentrations for 120 minutes. The upper graph shows the NO flux plotted against the concentration of S-nitrosothiols formed. The lower graph shows that the reaction appears to be closer to following second order kinetics with a higher correlation if the S-nitrosothiol concentration is plotted against the NO flux squared. All experiments n = 3. Data is shown as the mean ± s.e.m.

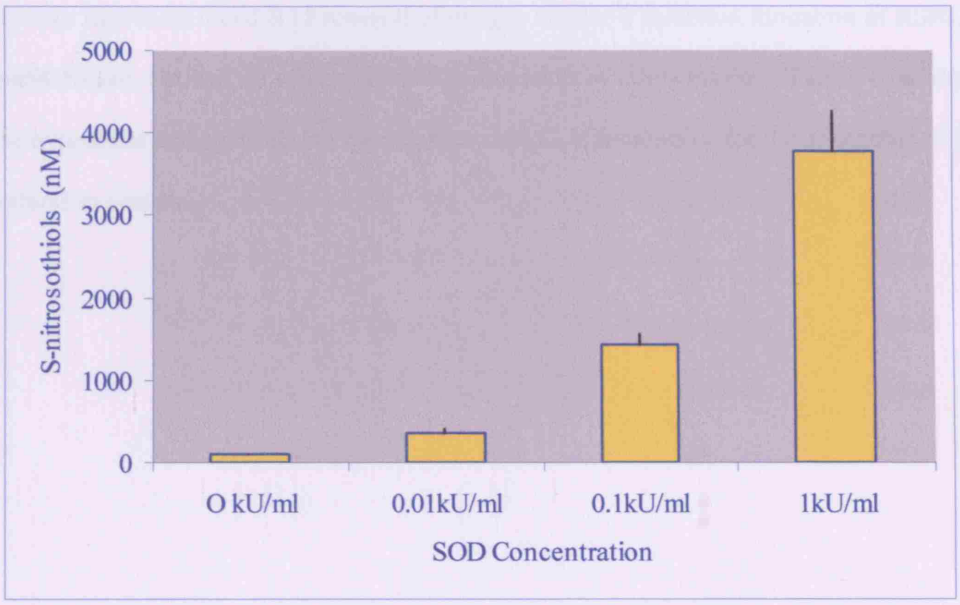


Figure 6.9 The effect of increasing concentrations of superoxide dismutase on the generation of S-nitrosothiols by SIN-1A (final concentration 50 μM). All experiments n = 3. Data expressed as mean ± s.e.m.

6.3.4 The Effect of Oxygen on the Generation of S-nitrosothiols

6.3.4.i Methods

The kinetic analysis of the formation of RSNO on exposure of NO to plasma under aerobic conditions is consistent with a reaction involving two molecules of NO and oxygen. To test this possibility, plasma was diluted 1 in 5 with 0.15M PBS, pH 7.4, and then sparged with helium for 30 minutes prior to and during a 30 minute incubation of plasma with 100 μM papaNONOate. The dilution with PBS was necessary to prevent excessive foaming due the sparging. The reaction was stopped by the addition of NEM (5mM) and the samples were then dialysed prior to assay. Control experiments using the same dilutions and concentration of papaNONOate were performed at atmospheric oxygen tensions. Identical experiments were performed using 250 μM peroxynitrite.

6.3.4.ii Results

As shown in figures 6.10 and 6.11 removal of oxygen markedly inhibited formation of RSNOs by papaNONOate, but had no effect on RSNO generation by peroxynitrite. This is consistent with the hypothesis that a nitrosating species such as N_2O_3 is responsible for the generation of S-nitrosothiols in plasma.

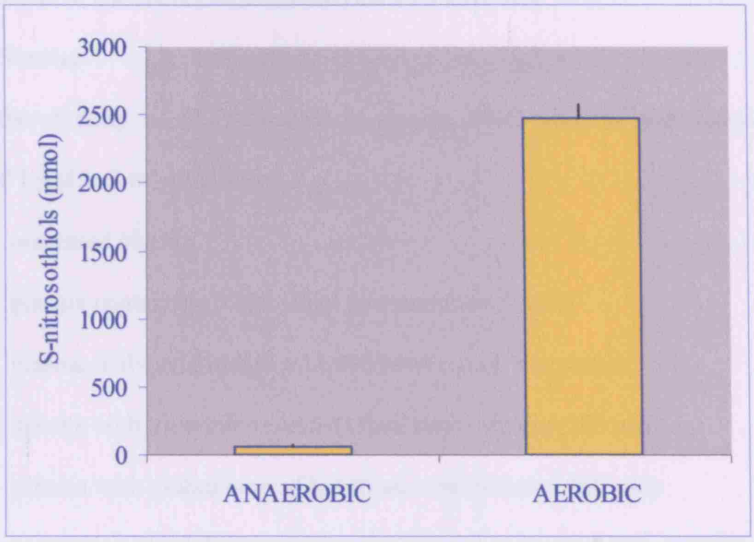


Figure 6.10 Anaerobic conditions prevent the formation of S-nitrosothiols when papaNONOate (100 μM) is incubated with plasma for 30 minutes (n = 3). Data shown is mean ± s.e.m

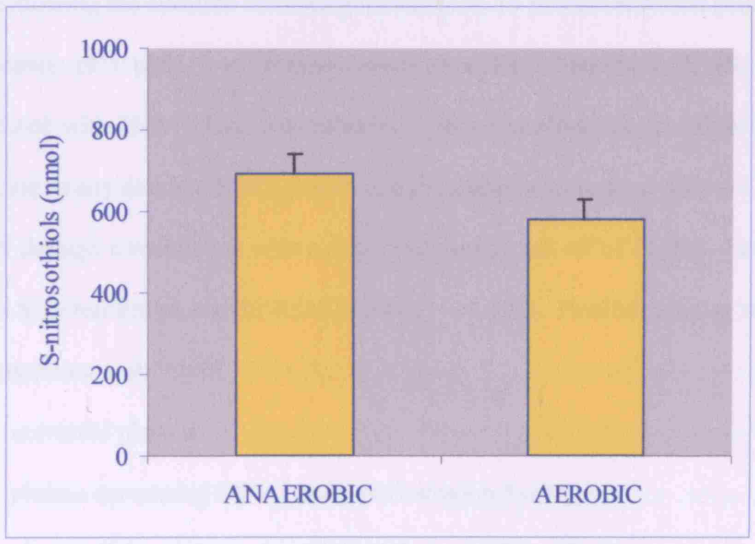


Figure 6.11 Anaerobic conditions had no effect on the formation of S-nitrosothiols following incubation of plasma with peroxynitrite (250 μM) for 30 minutes (n = 3). Data shown is mean ± s.e.m

6.4 THE STABILITY OF S-NITROSOTHIOLS IN PLASMA

6.4.1 Stability Studies

To determine the stability of SNO-albumin in plasma, SNO-albumin was added at a final concentration of 1 μM to 1 ml aliquots of

i) untreated plasma

ii) plasma containing NEM (final concentration 5 mM)

iii) plasma dialysed through a 14,000 Mwt cut off membrane

iv) plasma with glutathione added (final concentration 100 μM)

v) plasma with glutathione added (final concentration 500 μM)

All samples were prepared in triplicate and were incubated in a water bath at 37°C in the dark. 100 μl aliquots were removed at 0, 5, 15, 30, 60, and 120 minutes and then again at 24 hours for measurement of RSNO concentration.

The stability of SNO-glutathione, and its ability to transnitrosate to high molecular weight thiols was measured following the addition of SNO-glutathione to 10 mls of untreated plasma, to give a final concentration of 1 μM. 1 ml aliquots were removed at time 0, 2, 5, 10, 15, and 30 minutes and treated with NEM (final concentration 5 mM) to block the free thiol groups. In order to fully remove any residual SNO-glutathione the samples were then dialysed against 3 x 3 l PBS / DTPA through a membrane with a molecular weight cut off of 14,000. Samples were then assayed for high molecular weight RSNOs (Mwt > 14,000). Parallel samples were used to look at SNO-glutathione stability in

i) untreated plasma

ii) plasma containing NEM (final concentration 5 mM)

iii) plasma dialysed through a 14,000 Mwt cut off membrane

Aliquots were removed at the same time as for the SNO-albumin studies. The samples were then centrifuged through a 30,000 Mwt cut off filter and the RSNO concentration in the low molecular weight filtrate was assayed.

The effect of superoxide generation on SNO-albumin stability was also examined. SNO-albumin was added to plasma at a final concentration of 1 μM, either alone or in the presence of xanthine oxidase (0.75U/ml) and different final concentrations of hypoxanthine (0, 25, 100, 250, and 500 μM). Samples were left for 30 minutes in the dark at 37°C following which 100 μl aliquots were removed and the RSNO concentration determined. Again all experiments were performed in triplicate.

6.4.2 Results

6.4.2.i Stability of S-Nitrosoalbumin

As is shown in figure 6.12, SNO-albumin was relatively unstable in untreated plasma with 38% loss by 2 hours, and 81% loss by 24 hours at 37°C (result not shown). Addition of glutathione to plasma accelerated the decay of RSNOs. In the presence of 100 μM glutathione, 76% of the SNO-albumin signal was lost at 2hours, whereas in the presence of 500 μM there was none detectable at 60 minutes. Constituents of plasma with a molecular weight of less than 14,000 clearly contribute to the instability of SNO-albumin as the decay in dialysed plasma was only 22% at 2 hours compared to 38% in non-dialysed plasma. Moreover free thiols also contributed as SNO-albumin was stabilised by pretreatment of plasma with NEM with only 11% of the signal being lost at 2hours.

6.4.2.ii Stability of S-Nitrosoglutathione

The low molecular weight RSNO, SNO-glutathione was less stable than SNO-albumin with 69% of the detectable low molecular weight fraction being lost at within 10 minutes in untreated plasma as is shown in figure 6.13. The rate of loss was decreased by both pre-treatment with NEM and dialysis, with losses of 33% and 21% respectively.

6.4.2.iii Transnitrosation

Over the same period of time that the RSNO signal disappeared in the low molecular weight fraction in untreated plasma, RSNOs were detected in the high molecular weight fraction (*see*

figure 6.14) presumably as a result of transnitrosation between the thiol groups to compounds such as albumin. This transnitrosation was, however, only 30% efficient with the rest of the nitric oxide presumably diffusing into the gaseous phase or being converted to nitrite.

6.4.2.iv Effect of Superoxide

Finally the stability of SNO-albumin in plasma and in the presence of a superoxide generating system, was examined – see figure 6.15. In spite of increasing concentrations of hypoxanthine, in the presence of xanthine oxidase, the RSNO signal remained relatively stable with a recovery of greater than 90% of the control value for SNO-albumin in plasma alone, for all concentrations used.

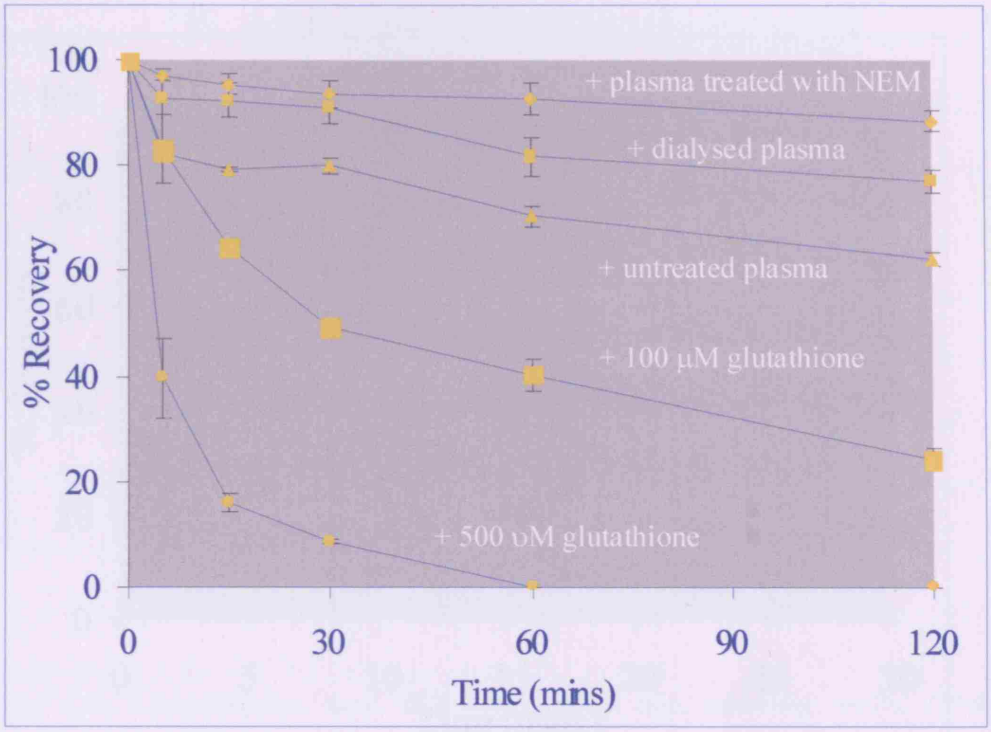


Figure 6.12 The stability of SNO-albumin in plasma. RSNO concentrations were assayed at varying times following addition to plasma. Figures are expressed as percentages of the initial signal. The presence of increasing concentrations of glutathione decreased the stability in plasma. Moreover dialysing plasma to remove compounds with a molecular weight of < 14,000 increased stability as did treatment with NEM (n = 3, all experiments). Data shown is mean ± s.e.m

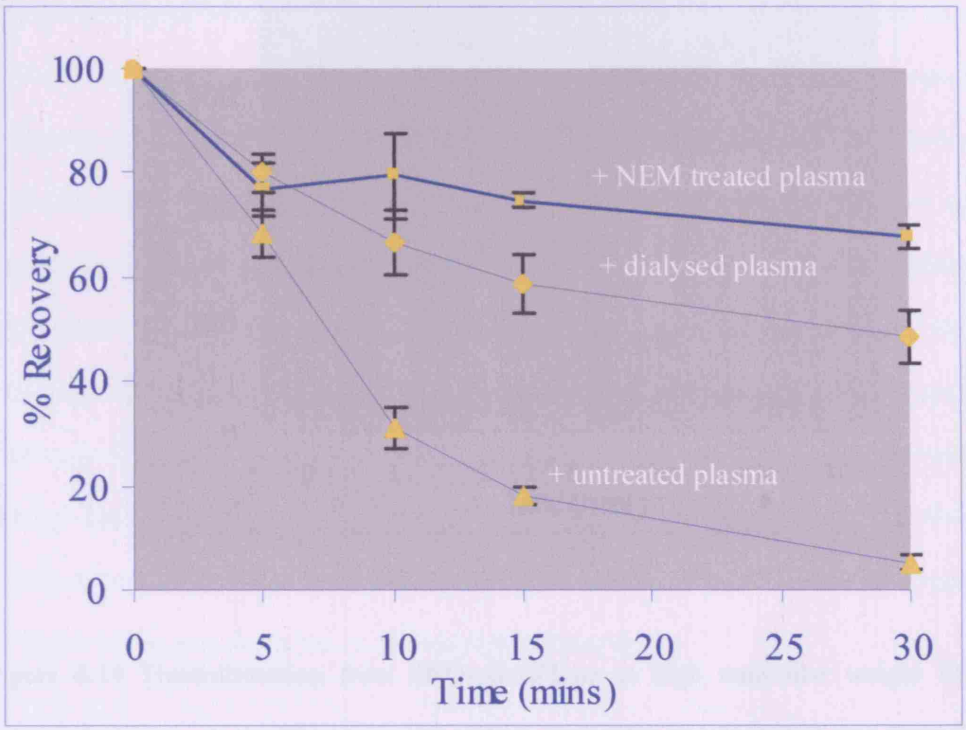


Figure 6.13 The stability of SNO-glutathione in plasma. As above RSNO concentrations were assayed at varying times following addition to plasma. Figures are expressed as percentages of the initial signal. Again, dialysing plasma to remove compounds with a molecular weight of < 14,000 increased stability as did treatment with NEM, compared with untreated plasma (n = 3, all experiments). Data shown is mean ± s.e.m

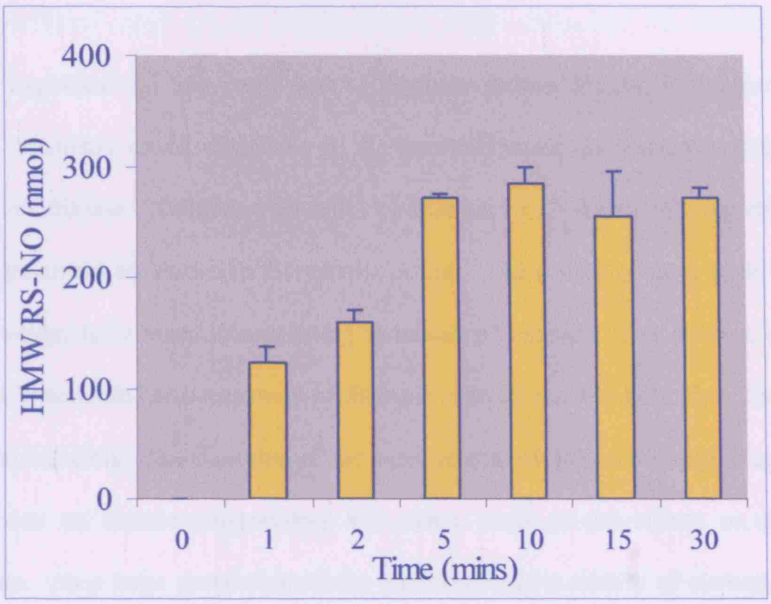


Figure 6.14 Transnitrosation from SNO-glutathione to high molecular weight RSNOs in untreated plasma. A significant amount of high molecular weight RSNO was formed when 1 μM SNO-glutathione was added to untreated plasma (n = 3). Data shown is mean ± s.e.m

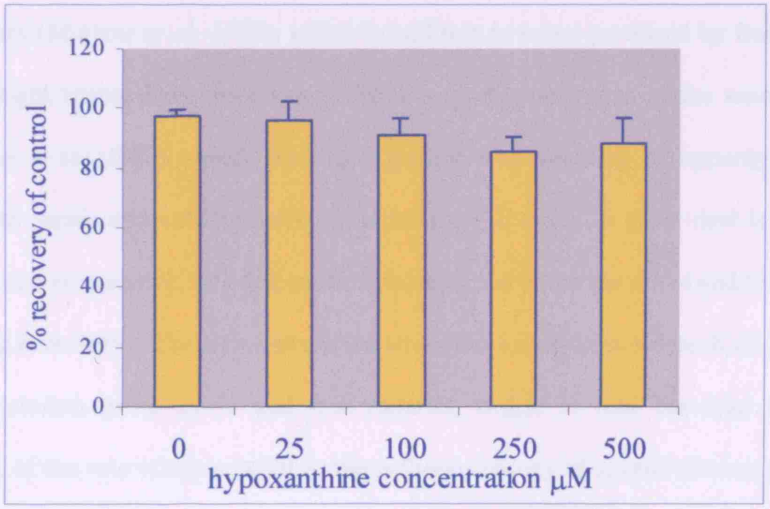


Figure 6.15 Increasing concentrations of hypoxanthine in the presence of xanthine oxidase, and therefore presumed superoxide generation, had no effect on the stability of SNO-albumin in plasma (n = 3). Data shown is mean ± s.e.m

CHAPTER 7 - DISCUSSION

The central hypothesis to this thesis was to explore whether oxidant injury and its effect on nitric oxide chemistry could contribute to the haemodynamic abnormalities that are found in advanced liver disease. Oxidative stress is ubiquitous in all forms of liver disease, making antioxidant treatment an attractive therapeutic option. The results of most trials of antioxidant therapy, however, have been disappointing in advanced cirrhotic liver disease, although there are potential benefits in early treatment of diseases such as non-alcoholic fatty liver disease and possibly viral hepatitis. The direction of the work presented differs from other approaches as it does not focus on disease progression, but rather looks at the effects on haemodynamic derangements. As a large proportion of the morbidity and mortality of cirrhotic liver disease results from vascular related complications such as variceal bleeding and ascites, arguably it is as important an area to analyse as disease progression *per se*.

In the decade prior to this research being undertaken there were two major discoveries which prompted this work. The first was the discovery of a group of products of lipid peroxidation, the isoprostanes (*Morrow et al. 1990*), which in addition to being produced by free radicals are in their own right vasoactive compounds. The second discovery was of the vasodilator nitric oxide (*Palmer et al. 1987*), which lead to a greater understanding of hyperdynamic states associated with sepsis and subsequently advanced liver disease. A great deal is now known about the enzyme responsible for nitric oxide synthesis, and about the direct and indirect effects of nitric oxide chemistry. The term nitrosative stress has arisen from research on the chemical interactions between nitric oxide and free radicals, which in turn has lead to a greater understanding of the role of nitric oxide in the pathophysiology of several disease processes, in particular related to vascular biology.

The work presented in this thesis has examined the role of isoprostanes in liver disease, the effects of oxidative stress on the production of nitric oxide and the chemistry of the S-nitrosothiols.

7.1 THE EFFECTS OF ISOPROSTANES – PRODUCTS OF LIPID PEROXIDATION – ON PORTAL HYPERTENSION

Central to the entire thesis was the hypothesis that oxidative stress occurred in liver disease. As outlined in the introduction, several previous studies have shown a depletion in endogenous antioxidants and an elevation in markers of oxidant stress in a wide variety of human liver diseases and animal models. It is now widely agreed that isoprostane measurement is the most sensitive assay of lipid peroxidation because of the high specificity and the lack of artefactual *ex vivo* generation which hampers other assays.

Measurement of the F_2-isoprostane 8-iso-$PGF_{2\alpha}$ using gas chromatography mass spectroscopy in both urine and plasma samples demonstrated that there were higher levels in patients with decompensated cirrhotics by comparison to normal controls. Moreover urinary levels were higher also in the compensated cirrhotics than in normals. It is likely that this increase is due to increased production, not reduced liver clearance, as previous studies have shown that the liver is poorly involved in the clearance of 8-iso-$PGF_{2\alpha}$ (*Roberts et al. 1996*).

There have been two published studies that have looked at the levels of F_2-isoprostanes in cirrhotic liver disease. In the first, Morrow *et al* (*1993*) showed that the total level of plasma isoprostanes was significantly higher in patients with the hepatorenal syndrome, but not in compensated or decompensated cirrhotics. Measurement of levels of urinary F_2-isoprostanes in timed urine collections offers an advantage over plasma samples in that it can provide an integrated index of isoprostane production over time, and therefore a more accurate reflection of ongoing oxidant injury. In order to correct for variations in renal function the concentration is expressed as a ratio of the absolute concentration in urine to a measurement of renal function,

in this case creatinine clearance. Pratico *et al* (*1998a*) have also shown elevated urinary levels of 8-iso-$PGF_{2\alpha}$ in cirrhotic patients. They expressed there data in a different way comparing medians of all cirrhotics with a normal control group, and then showing that a higher percentage of patients with Childs C cirrhosis had levels above the upper limit of normal than both Childs A and B patients.

The source and the stimulus for this increase in urinary isoprostane production is not clear. It may reflect the depletion of endogenous antioxidants that is seen in liver disease, or the ongoing inflammatory or toxic process that have lead to the development of cirrhosis. Elevated renal production cannot be completely excluded, however would seem unlikely. The pathogenesis of renal impairment in decompensated cirrhosis and patients with ascites is primarily functional and due to reduced renal blood flow rather than renal pathology, and as such is less likely to be asociated with oxidative stress. An interesting observation from the study by Pratico *et al* (*1998a*) was that the urinary isoprostane concentration was proportional to the circulating endotoxin levels. This may be just a reflection of the severity of the liver disease, as increased endotoxin levels occur with progressive decline in liver function, or may be a stimulus for lipid peroxidation.

Having confirmed that isoprostane production was increased in liver disease, the next part of the work presented looked at the physiology of one of the most abundant F_2-isoprostanes, 8-iso-$PGF_{2\alpha}$. Infusion of 8-iso-$PGF_{2\alpha}$ into the isolated perfused rat liver leads to an increase in portal pressure. There was a marked hyperresponsiveness in rats that had undergone bile duct ligation, leading to cirrhotic change in the liver, when compared to normal rats. The study also confirmed the observation by Fisher *et al* (*1987*) that U46619, a thromboxane agonist, increases portal pressure in the normal rat, and also extends this observation to the cirrhotic animal. As observed with 8-iso-$PGF_{2\alpha}$, there was increased responsiveness to U46619 in the group of cirrhotic animals. This work is consistent with the studies showing that 8-iso-$PGF_{2\alpha}$ is a potent

vasoconstrictor in both the renal and pulmonary circulations (*Takahashi et al. 1992, Kang et al 1993*).

There is good *in vitro* evidence for the presence of thromboxane receptors in the portal vasculature. Infusion of U46619 into normal rat livers increases both glucose and oxygen consumption, as well as increasing portal pressure (*Fisher et al. 1987*) and radioligand binding studies have revealed thromboxane receptors on hepatic sinusoidal endothelial cells (*Ishiguro et al. 1994*). Antagonism of the portal pressure response to 8-iso-$PGF_{2\alpha}$ by SQ29548 shows that 8-iso-$PGF_{2\alpha}$ acts via the thromboxane or a thromboxane like receptor. From the simple infusion studies described it is not possible to state whether 8-iso-$PGF_{2\alpha}$ acts as a partial thromboxane agonist or whether it acts on an independent but closely related receptor. Two structurally similar but pharmacologically different receptors, for thromboxane and 8-iso-$PGF_{2\alpha}$, may explain why the response to U46619 is more potent than 8-iso-$PGF_{2\alpha}$.

Having made the observation that a product of lipid peroxidation was capable of causing vasoconstriction in the portal circulation the next step was to determine at which level 8-iso-$PGF_{2\alpha}$ was acting and to determine whether or not this observation was likely to be of any clinical relevance.

It is well recognised that liver injury and cirrhosis is associated with the transformation of hepatic stellate cells into myofibroblasts (*Horn et al. 1985*), which express a variety of contractile proteins including α smooth muscle actin and desmin. These cells are closely related to sinusoidal vessels and the degree to which they contract is likely to modify vascular resistance within the liver and therefore contribute to portal pressure in liver disease. The exaggerated response seen in the cirrhotic animals to both 8-iso-$PGF_{2\alpha}$ and the thromboxane recptor agonist U46619 may therefore be secondary to receptor expression on these myofibroblasts.

In order to test the hypothesis that 8-iso-$PGF_{2\alpha}$ mediated vasoconstriction was secondary to stellate cell contraction, work was carried out in the University of California, San Francisco, with an isolated stellate cell contractility assay. Although endothelin-1 has been shown to mediate contraction with this system (*Rockey and Weisiger, 1996*), there was no effect with exogenously applied 8-iso-$PGF_{2\alpha}$, even at concentrations higher than those used in the infusion studies. This would suggest that the vasoconstrictive effect is not mediated by myofibroblasts. This may reflect the use of stellate cells isolated from rats that had undergone bile duct ligation only 14 days previously, whereas those used in the perfusion studies were examined between days 25 and 28, or that a different level in the vasculature was responsible. The results were surprising as previous studies by Kawada *et al* (*1992*) had shown that U46619 leads to stellate cell contractility, albeit in a slightly different assay system.

Another potential site where 8-isoprostaglandin-$F_{2\alpha}$ may be acting is at the level of the portal vein, or portal venous radicles. Attempts were made to isolate portal veins and perform organ bath contractility studies. However, it was not possible to obtain satisfactory dose response curves in control experiments, and these experiments were abandoned. Clearly further work needs to be carried out to elucidate how 8-iso-$PGF_{2\alpha}$ causes vasoconstriction, and such work would include ligand binding studies and perhaps repeating the stellate cell contractility studies but using animals between 25 and 28 days following bile duct ligation. Moreover, up to 64 F_2-isoprostanes are formed, and similar families of isomers of other prostaglandins and lipooxygenase products are likely to be generated. This level of complexity constrains the interpretation of experiments conducted with a single isomer, especially if trying to interpret their role as autocoids *in vivo*.

Following the publication of the results of the infusion studies (*Marley et al. 1997*) a study was published by Pratico *et al* (*1998b*) which specifically questioned the relevance of these findings. In this study patients undergoing elective transjugular intrahepatic portosystemic shunting (TIPS) for chronic variceal bleeding had blood sampled from the systemic circulation and the

portal vein. Levels were significantly higher in the portal vein, implying increased production in the splanchnic bed, however there was no correlation between portal pressure and portal venous levels of 8-isoprostaglandin-$PGF_{2\alpha}$. The authors argued that isoprostanes were therefore unlikely to be of significance in portal hypertension. Indeed, the concentration of 8-iso-$PGF_{2\alpha}$ required to elicit a significant increase in portal pressure is approximately one order of magnitude higher than those found in experimental liver injury secondary to carbon tetrachloride and alcohol (*Morrow et al. 1992, Nanji et al. 1994*).

It could be argued that the lack of correlation between circulating isoprostane levels and portal pressure is not surprising, especially as in stable liver disease at most intrahepatic resistance only contributes to 15% of the total portal pressure. Portal pressure is unlikely to be a static parameter, but rather subject to acute surges. The factors that precipitate variceal bleeding in patients with portal hypertension are still poorly defined. There is good evidence that variceal haemorrhage occurs more frequently during periods of sepsis (*Bernard et al. 1995*), in particular spontaneous bacterial peritonitis, and some evidence in patients who acutely consume large quantities of alcohol (*Poynard et al. 1987, Colombo et al. 1989*). Both alcohol and sepsis are oxidant stressors and lead to lipid peroxidation. It may be that acute production of F_2-isoprostanes, in these circumstances, may be linked to surges of portal pressure which precipitate variceal bleeding.

In work carried out in the laboratory and published, but not included in this thesis, the response to boluses of endotoxin was examined in bile duct ligated cirrhotic rats (*Harry et al. 1999*). In this study, following injection of low dose lipopolysaccharide intraperitoneally into BDL rats and normal rats there was a rapid 6 fold increase in plasma 8-iso-$PGF_{2\alpha}$ levels, whereas in the control rats there was a more modest two-fold increase. This may represent poorer host antioxidant defences in the cirrhotic animals, or possible upregulation of phospholipase A_2 which cleaves isoprostanes from their *in situ* formation on cell membranes. Such a sudden rise

in the concentration of 8-iso-$PGF_{2\alpha}$ may be expected to cause intense vasoconstriction in the intrahepatic portal circulation and may provide a link between sepsis and variceal bleeding.

As mentioned above, studies of variceal bleeding have also demonstrated an indirect link between ethanol consumption and variceal bleeding, though one study specifically looking at this failed to show a definite association (*McCormack et al. 1992*). The effect of ethanol on hepatic haemodynamics has been examined in both cirrhotic and non-cirrhotic humans. Oral consumption of alcohol in patients with cirrhosis leads to a rise in the hepatic venous pressure gradient, an indirect measure of portal pressure (*Luca et al. 1997*). These findings are at odds with a study by Ready *et al.* (*1990*) who failed to demonstrate a rise in portal pressure following alcohol administered intravenously, but giving similar plasma levels as the Luca study. This difference probably relates to the mode of ethanol administration. Oral administration has been shown to give double the portal vein ethanol concentration as the intravenous route (*Beck et al. 1974*). Another study looking at intravenous ethanol in non-cirrhotic alcoholics also demonstrated a rise in portal pressure (*Silva et al. 1994*). To further test the hypothesis that acute oxidant stressors could lead to an isoprostane mediated surge in portal pressure, ethanol was infused into the portal vein of isolated livers of control rats and rats with cirrhosis secondary to bile duct ligation. Ethanol has been documented in numerous studies to cause lipid peroxidation and 8-iso-$PGF_{2\alpha}$ production. Infusion of ethanol into the portal vein caused a much greater portal pressure response in perfused cirrhotic livers than in perfused normal livers, a similar finding to the response to 8-iso-$PGF_{2\alpha}$ and U46619. Moreover these findings occured using ethanol concentrations that occur in the portal vein following heavy alcohol consumption (*Hamlyn et al. 1975*). The effect of thromboxane receptor blockade was however disappointing with no significant reduction in the portal pressure response, making it unlikely that this was a direct isoprostane mediated phenomenon.

Previous studies in normal rats have shown that ethanol causes endothelin mediated intrahepatic vasoconstriction at the level of the sinusoids (*Oshita et al.* 1993). Having demonstrated an

exagerated response in cirrhotic rats and as stellate cells express increased amounts of endothelin receptors as a part of their transformation into myofibroblasts (*Pinzani et al. 1996*), the effects of bosentan were examined. Bosentan is a combined endothelin A and B receptor antagonist and its co-infusion with ethanol significantly reduced the intrahepatic vasoconstriction and portal pressure rise in cirrhotic animals. Although there is evidence that ethanol causes endothelin release there have been no direct studies to look at whether this is secondary to oxidant stress or a direct effect on endothelin synthesis itself. A role for F_2-isoprostanes seems unlikely as thromboxane receptor blockade had no effect on the response to ethanol. However, 8-iso-$PGF_{2\alpha}$ does cause a dose dependent increase in endothelin mRNA and endothelin-1 production in cell culture systems (*Yura et al. 1999*) and antioxidant treatment with vitamin E treatment reduces endothelin production (*Martin-Nizard et al. 1998*).

There are disadvantages at looking at an isolated non-recirculating perfused liver system. The time course of the experiments can only look at the effects of different doses over short periods of time. The system is *ex-vivo* and as there is no recirculation factors produced by the liver itself as well as other organ systems are ignored. Using a flow rate of 25 ml/min in cirrhotic rats may have been an underestimate of the actual flow rate that is found as a consequence of splanchnic vasodilatation. Future directions may be to look at blood flow in the sinusoidal circulation or the portal vein with non-invasive doppler probes, in anaesthetised rats, and then examine the effects of alcohol administration on liver blood flow. It may be that thromboxane and endothelin receptor antagonists will become the future focus of research into treatment of portal hypertension and prevention of variceal bleeding in at risk groups.

7.2 THE EFFECTS OF LIPOIC ACID ON THE HYPERDYNAMIC CIRCULATION

A role for oxidant stress in the development of the hyperdynamic circulation was first proposed on the basis of work carried out in the laboratory, but not included in this thesis (*Fernando et al. 1998*). This hypothesis was based upon our observations in rats that had undergone portal vein stenosis and were injected with the GSH precursor N-acetylcysteine twice daily for 14 days.

Untreated rats rapidly developed presinusoidal portal hypertension and a hyperdynamic circulation, which was associated with enhanced oxidative injury as demonstrated by an increase in urinary F_2-isoprostane excretion. Treated rats did not develop a hyperdynamic circulation, experienced a reduction in portal hypertension, and the enhanced urinary excretion of F_2-isoprostanes was suppressed. Moreover, this was associated with a reduction in plasma nitrite and nitrate, though the effect on nitric oxide synthase activity was not specifically examined. Hepatocellular injury is not a feature of this animal model of portal hypertension, although a hyperdynamic circulation does develop. This data suggested that there was a causal link between oxidative stress and the hyperdynamic circulation. It also suggested that in the absence of liver injury the oxidative stress may originate from the portal circulation itself.

The chronic administration of lipoic acid prevented the development of the hyperdynamic circulation and attenuated the rise in portal pressure in biliary cirrhotic rats. The potential mechanisms examined were the effects of lipoic acid on histopathological and biochemical parameters, and its effect on lipid peroxidation and NO production. Lipoic acid has been described as the universal antioxidant, and it was chosen for this study because of its numerous biochemical actions including free radical scavenging, regeneration of endogenous antioxidants such as glutathione and vitamin E, and its ability to inhibit upregulation of NFκB. It is also readily taken up by the liver following oral administration. The dose used was based upon other published work from the laboratory, which is not included in this thesis (*Holt et al. 1999*). In this work lipoic acid administration almost completely blocked urinary isoprostane generation following acute bile duct ligation – a model of renal dysfunction.

Previous studies using S-adenosylmethionine and N-acetylcysteine have shown histological improvement following bile duct ligation (*Muriel et al. 1994, Pastor et al. 1997*). Despite a significant increase in hepatic reduced glutathione levels, lipoic acid did not appear to have any effect on histological progression of the disease. This is in keeping with a human study showing no effect in the treatment of alcoholic liver disease (*Marshall et al. 1982*). It may be a

reflection of the dose used or alternatively S-adenosylmethionine and N-acetylcysteine may have additional properties over and above those of lipoic acid.

Portal pressure is a product of portal venous blood flow and vascular resistance. Resistance to flow is predominantly due to structural changes in the liver. However, up to 15% of the resistance may be due to changes in the intrahepatic circulation, due to changes in sinusoidal tone (*Bhathal & Grossman. 1985*). Having previously demonstrated that 8-isoprostaglandin-$F_{2\alpha}$ causes an increase in portal pressure it was a mistake not to have measured this compound in this particular study. Particularly as lipoic acid had been shown in other studies to reduce 8-iso-$PGF_{2\alpha}$ following acute bile duct ligation (*Holt et al. 1999*) and that N-acetylcysteine reduced levels in the partial portal vein ligated model (*Fernando et al. 1998*). Lipid peroxidation was measured instead by looking at levels of the reactive aldehydes malonaldehyde and 4-hydroxynonenal, levels of which were significantly increased in the untreated group with secondary biliary cirrhosis. Of interest, whereas lipoic acid significantly suppressed levels in normal rats, it had no significant effect in the BDL cirrhotic animals. The active metabolite of lipoic acid, dihydrolipoic acid, protects against lipid peroxidation by enabling recycling of the antioxidant, vitamin E (*Scholich et al. 1989*). Following bile duct ligation, plasma and hepatic levels of vitamin E are significantly decreased (*Singh et al. 1992*). Thus, the decreased effect of lipoic acid in biliary cirrhosis may be secondary to the low levels of endogenous vitamin E.

The other component to portal pressure is splanchnic blood flow. Having demonstrated an overall reduction in the development of the hyperdynamic circulation it is likely that this is the major contribution to the reduction in portal pressure that was observed. Several mediators have been implicated in the development of the hyperdynamic circulation of cirrhosis. As outlined in the introduction to this thesis most interest has focused on NO in recent years. In this study, the development of biliary cirrhosis was accompanied by an increase in total hepatic NOS activity and an increase in plasma nitrite/nitrate. These changes were both prevented by administration of lipoic acid.

As outlined in chapter 1 there is a great deal of controversy as to which isozyme of NOS is responsible for the increased production of NO in both animal models and human cirrhotic liver disease. In this study, following chronic bile duct ligation hepatic NOS activity was significantly upregulated by comparison to controls. This confirms work from a previous study (*Kanwar et al. 1996*) which showed increased iNOS and eNOS activity in livers from bile duct ligated rats. It was not found possible to get meaningful results using aortic tissue using the assay employed, in part due to the small quantities involved.

In spite of the relative lack of consensus regarding the relative contribution of both isoforms to NO production, there is evidence to suggest that both may be upregulated by oxidative stress and be inhibited by antioxidants. One of the transcription factors important in activating iNOS gene expression is nuclear factor κ B, and activation of nuclear factor κ B is strongly inhibited by lipoic acid (*Flohe et al. 1997, Suzuki et al. 1992*). Several antioxidants reduce iNOS expression in response to lipopolysaccharide *in vitro*, (*Duval et al. 1995, Galley et al. 1996*) and *in vivo*, vitamin E analogues reduce the vascular hyporeactivity observed in lipolysaccharide-treated rats (*Loegering et al. 1995*). The evidence for oxidative stress leading to upregulation of eNOS is less convincing. eNOS activity is increased in response to hypoxia (*Xue et al. 1994, Gess et al.1997*) and shear stress (*Noris et al. 1995*), both of which may be important at a cellular level in cirrhosis. The former leads to the generation of free radicals by mitochondria, and the latter is transduced via extracellular-regulated kinases (*Takahashi et al. 1997*), which themselves can be activated by reactive oxygen species (*Guyton et al. 1996*) and inhibited by antioxidants (*Wilmer et al. 1997*).

In conclusion, this study demonstrates that chronic administration of lipoic acid prevents development of the hyperdynamic circulation and upregulation of nitric oxide synthase activity following bile duct ligation. There was, however, no benefit of lipoic acid on disease progression as measured by biochemical or histological parameters. Further studies are

necessary to specifically examine the effect of lipoic acid on the two isoforms of NOS, and more importantly, to determine whether lipoic acid can reverse the hyperdynamic circulation after liver injury has been established.

7.3 S-NITROSOTHIOL ASSAY DEVELOPMENT AND CHEMISTRY

Liver disease is associated with high levels of NO production, probably as a consequence of iNOS upregulation. Also, as demonstrated in this work, it is associated with increased free radical production and oxidative stress. Theoretically this biochemical environment would favour the indirect effects of nitric oxide and therefore the generation of compounds such as S-nitrosothiols.

There has been indirect evidence to suggest that S-nitrosothiols may circulate in physiologically relevant concentrations in liver disease. Three studies have looked at the haemodynamic consequences of intravenous infusion of N-acetylcysteine into patients with liver disease. Two of these studies looked at patients with fulminant hepatic failure. In one there was a significant fall in systemic vascular resistance after a 30 minute NAC infusion (*Harrison et al. 1991*), in the other there was also a fall at one hour, but this did not reach statistical significance (*Walsh et al. 1998*). Similarly in a study of patients with a spectrum of cirrhotic liver disease, a short N-acetylcysteine infusion also lead to a significant fall in SVR (*Jones et al. 1994*). This effect may be due to transnitrosation reactions occuring as a result of infusing a low molecular weight thiol, leading to NO release in a similar fashion to the original description of Scharfstein *et al* (1994) showing cysteine enhanced the hypotensive effect of SNO-albumin in rabbits.

The main limitation to studying the role of S-nitrosothiols in biology and pathophysiology at the time that this work was performed was the lack of a reliable assay for their measurement in biological samples. A large amount of the work included in this thesis describes a novel assay for the measurement of plasma S-nitrosothiols, which has the advantage of being simple, highly sensitive and reproducible, and suitable for the analysis of a large number of samples within a

short period of time. In particular, it has been established that background nitrite can be removed quantatively by reaction with sulphanilamide / hydrochloric acid after thiol groups have been blocked by NEM without affecting the subsequent breakdown of RSNOs to NO. Cleavage of the S-NO bond is achieved by reaction with a mixture consisting of copper (I) / iodine / iodide, and the NO which is generated in high yield is then detected by its chemiluminescent reaction with ozone. A reliable method for the removal of interfering nitrite, as described in the present study, represents a major methodological advancement in the quantatitive analysis of low concentrations of RSNOs, since this is a common problem with most if not all other assays. This, together with the demonstration that SNO-albumin added to plasma and blood can be stoichiometrically recovered enabled reliable determination of the concentration of endogenous RSNOs in humans. This assay has now been widely adopted worldwide in further research on S-nitrosothiols.

The previous demonstration that alkylation of thiol groups by NEM stabilises RSNOs in plasma by preventing transnitrosation reactions (*Scorza et al. 1997*) and that metal ions, even at low concentrations, can accelerate RSNO degradation (*Gorren et al. 1996*) lead to blood being collected onto solutions containing both NEM and EDTA. With this regime exogenous SNO-albumin added to whole blood was recovered almost quantitatively and could readily be detected down to low nanomolar concentrations.

No attempts have been made to determine the exact mechanism by which RSNOs are cleaved to NO by the reagent mixture employed in this study. However, addition of copper (II) sulphate to an acidic solution of potassium iodide will result in the oxidation of iodide (I^-) to iodine (I_2), and – assuming stoichiometric oxidation of iodide by copper (II) – it is hypothesised that this redox reaction results in a mixture of iodine / iodide and Cu^+ ions at approximately 40 / 60mM and 40mM, respectively. This notion is supported by the observation that the reactant turns brown immediately on addition of the copper (II) solution, which is characteristic of aqueous solutions of I_3^- formed from complexation of I_2 by I^-. In view of the fact that both Cu^+ ions and free

iodine have been shown to effectively generate NO from RSNOs the following sequence of chemical reactions is proposed to account for the observed stoichiometric cleavage of RSNOs to NO:

$$I_2 + I^- \rightarrow I_3^-$$

$$I_3^- + 2RSNO \rightarrow 3I^- + RS^* + 2NO^+$$

$$2NO^+ + 2I^- \rightarrow 2NO + I_2$$

$$RSNO \rightarrow RS^* + NO$$

$$2RS \xrightarrow{Cu^{++}} RSSR$$

As described, a method based upon removal of background nitrite by sulphanilamide under acidic conditions and back titration to neutral pH, followed by measurement of the increase in nitrite following addition of mercuric or cuprous ions, was extensively evaluated. Although this method produced perfect standard curves in buffer, the slopes of the standard curves obtained from spiking experiments of plasma with known amounts of authentic SNO-albumin showed considerable inter-individual variations and poor reproducibility. It appeared that mercury did not stoichiometrically release NO from RSNOs when added to plasma, and this was related to the total concentration of thiols present in the plasma. This represents a general drawback of the classical Saville reaction. A more serious problem using this approach is observed in samples containing both nitrite and thiols, since thiols may compete with sulphanilamide for reactions with nitrite leading to artefactual S-nitrosation when thiol concentrations are relatively high (e.g. plasma). Although this problem can be avoided by prior alkylation of free thiol groups (as described in this study) this potential pitfall appears not to be widely recognised.

Samouilov and Zweier (*1998*) have tried to address the problem of contaminating nitrite in biological fluids. They also evaluated the efficacy of ammonium sulphamate for the elimination

of nitrite, and observed greater efficiency of nitrite elimination than was observed in the current study. For the determination of S-nitrosothiols they compared two methods, namely the measurement of nitrite and nitrite plus S-nitrosothiols under acidic conditions, with subtraction of the level of nitrite from the total concentration. This method has the advantage of being carried out under acidic conditions in which the S-nitrosothiols are relatively stable. However, methods that rely on measuring differences of nitrite in the presence of a relatively high background level are more likely to suffer from problems of accuracy and reproducibility than those offering a direct approach, unless the concentration of S-nitrosothiols is high. Their second approach was an alkaline method based on reaction with hydroquinone / quinone. This method worked well for the release of NO from S-nitroso-glutathione or S-nitroso-penicillamine, but no data was given for S-nitrosoalbumin. This study did not report the concentrations of S-nitrosothiols in plasma.

In conclusion, by combining a reagent mixture comprised of copper (I) / iodine / iodide for the specific and effective cleavage of RSNOs, and a reaction chamber, which allows large volumes of plasma to be assayed, with a sensitive chemiluminescence technique to detect NO, the stoichiometric removal of endogenous nitrite by sulphanilamide, and the stabilisation of RSNOs by NEM and EDTA, this method allows the reliable quantification of low nanomolar concentrations of endogenous RSNOs in complex biological matrices.

There have only been limited references to the levels of circulating plasma S-nitrosothiols, reinforcing the limitations of previous assays. The initial measurement of 7 μM (*Stamler et al.* 1992) using photolysis, is widely recognised as a gross over estimate, although a recent publication quoted even higher levels of 9.2 μM (*Tyurin et al. 2001*). The Stamler figure is likely to be due photolytic release of NO from other compounds such as nitrite, nitrosyl iron complexes and nitrosamines (*Alport et al. 1997*).

An assay based upon Hg^{2+}-liberated nitrite followed by a vanadium (III) reduction to NO and subsequent chemiluminescent quantification (*Ewing and Janero 1998*) measured plasma S-nitrosothiol levels of approximately 320 nM, however they failed to measure plasma nitrite levels which would also release NO under the conditions applied. Finally, a method employing an HPLC step with the classical Saville method calculated plasma levels to be approximately 220 nM (*Goldman et al. 1998*). This method only has a sensitivity of 100 nM and is dependant upon subtraction of background nitrite levels away from the total to give an estimated level.

The total S-nitrosothiol concentration levels in healthy human plasma, measured by the assay developed in this work, was 28 ± 7 nM. This figure correlates closely with the estimated concentration based upon the concentration-response relationship of the synergism between NO and hydrogen peroxide in inhibiting platelet concentration (*Naseem et al. 1996*). A recent study of the response to inhaled NO in healthy human volunteers (*Cannon et al. 2001*) using a similar chemiluminescens based assay has given a similar venous plasma level of 35 ±13 nM.

Due to the constraints of time, measurement of plasma S-nitrosothiol concentrations in patients with cirrhosis and rats with biliary cirrhosis was not undertaken, although in acute bile duct ligation there was a significant elevation in circulating levels. Experiments performed subsequent to the work presented in this thesis did look at circulating levels of RSNOs in rats with secondary biliary cirrhosis (*Ottesen et al. 2001*). This showed that there was a significant increase in BDL rats (206 ± 59 nM vs. 51 ± 6 nM in control rats). In order to confirm that this was due to increased synthesis rather than reduced elimination the plasma clearance of S-nitrosoalbumin was compared between the two groups and was not found to be statistically different. Furthermore there was a marked increase in plasma concentrations following the intraperitoneal injection of endotoxin. Levels in the cirrhotic rats increased from 206 ± 59 nM to 1335 ± 423 nM at two hours ($P < 0.001$), whereas the increase in control rats, though still significant was more modest, from 51 ± 6 nM to 108 ± 23 nM ($P < 0.01$). Future work would

involve the quantitation of plasma s-nitrosothiols in patients with liver disease, with correlation with haemodynamic parameters and degree of decompensation.

Having established a reliable assay for the detection of plasma S-nitrosothiols the next step was to use this assay to examine the chemistry of S-nitrosothiols in plasma, in order to further understand their role in health and liver disease. Both the the biochemical pathways leading to RSNO generation and th effects of low molecular weight thiols on RSNO stability were studied.

The significant formation of S-nitrosothiols *in vivo*, by the reaction of oxygen and NO is controversial (*Gaston 1999, Beckman and Koppenol 1996*). It has been suggested that in view of the low concentration and short life span of NO *in vivo* its reaction with oxygen to form a nitrosating species is too slow to account for any detectable amount of circulating RSNO.

This study, however, confirmed that under aerobic conditions NO is capable of generating S-nitrosothiols in plasma. Incubation of plasma with papaNONOate, a NO donor with a relatively short half life, lead to the production of compounds which gave rise to the release of NO under the reaction conditions described for RSNOs. The finding that treatment of plasma with NEM, a thiol blocking agent, prior to incubation with papaNONOate almost completely abolished the signal suggests that the formed compounds are indeed RSNOs. Further confirmatory evidence of their nature comes from the observation that the signal can be largely attenuated by addition to the nitrosated sample of 0.1% mercuric chloride prior to addition of sulfanilamide / HCl, a treatment which is known to destroy RSNOs.

The endothelium is constantly producing NO at a rate of 1 – 4 nM/s, although under pathological conditions this is thought to increase some 10 – 20 fold (*Moellering et al. 1999*). In order to try and mimic *in vivo* conditions similar flux rates were reproduced. The long half-life NO donor detaNOate was chosen and incubated with plasma over a concentration range of 0

to 1000 μM. Work performed in our laboratory, using chemiluminescense detection of NO release lead to a calculated steady state NO release of 0 – 60 nM/s in buffer, which is in the range of physiological and pathological production at the endothelial surface – and these figures are therefore included in chapter 6. Subsequently, however, work from the collaborating laboratory of Dr. Darley Usmar, using a more precise method with a NO electrode measured rates of NO release in plasma and found them to be 10 fold lower than those observed in buffer.

DetaNONOate lead to an accumulation of RSNOs at a constant rate in plasma over the two hour time period studied. Increasing the concentration of detaNONOate resulted in a progressive increase in RSNO formation. This relationship was linear if plotted as a function of the square of the rate of NO formation, and is therefore consistent with a reaction of two NO molecules and oxygen to form a nitrosating agent such as N_2O_3. As a dialysis step was essential in order to remove the NO donor prior to analysis for RSNOs these experiments could not determine the proportion of RSNO formed in the low and high molecular weight fractions of plasma. This problem was overcome by using an aqueous solution of NO. This experiment showed that both high and low molecular weight thiols can be S-nitrosated in plasma by reaction with NO or inter-converted by transnitrosation reactions. The greater concentration of high molecular weight RSNOs reflects the fact that high molecular weight thiols predominate in plasma, almost exclusively as albumin. Indeed albumin was shown to be the major site for S-nitrosation by selective affinity chromatography.

As noted above, the kinetic analysis of the formation of RSNO on exposure of NO to plasma under aerobic conditions is consistent with a reaction involving two molecules of NO and oxygen. This was confirmed by sparging plasma with helium to remove the oxygen, which in turn markedly inhibited formation of RSNOs by papaNONOate.

A recent study has also addressed the issue of how NO can generate S-nitrosothiols in view of the slow reaction kinetics (*Rafikova et al. 2002*). NO and oxygen are both hydrophobic

molecules, such that areas of high hydrophobicity should act to increase their local concentration by sequestering them from the surrounding aqueous phase, and therefore lead to increased formation of nitrosating species such as N_2O_3. One such hydrophobic molecule is albumin. This study demonstrated that the concentration of NO inside the albumin globule would be $\approx$ 120 times higher than that of the surrounding aqueous phase leading to significant RSNO generation.

A major contribution to the short half-life of NO and hence its chemistry *in vivo* is haemoglobin. The work outlined above was performed in erythrocyte free samples. As a continuation of the work in our laboratory, not included in the thesis, whole blood was incubated with varying doses of detaNONOate, prior to centrifugation, dialysis and assay for plasma S-nitrosothiols. The presence of red blood cells (and therefore erythrocyte haemoglobin) caused a marked inhibition of the formation of S-nitrosothiols in plasma, presumably due to scavenging of NO by the haemoglobin. This experiment is not wholly representative of *in vivo* conditions as during blood flow a red-cell-free zone exists at the endothelial cell surface, with reduced haemoglobin scavenging in this part of blood vessels (*Liao et al. 1999*). Even in the absence of this flow effect, a NO flux of 1.4 nMs^{-1}, which is in the physiological range, in the presence of red blood cells lead to the formation of mean plasma S-nitrosothiol concentration of 77 nM (*Marley et al. 2001*). This is comparable to the mean concentration of 28 nM found in healthy subjects in the earlier study.

Although analysis of RSNO generation in plasma and blood *ex vivo* represented a significant advance over work performed prior to this using buffer solutions, there are still limitations in its interpretation. As outlined above flow characteristics in blood vessels mean that the area adjacent to the endothelium, where NO is produced, tends to be erythrocyte free. It would be very difficult to mimic these conditions in the laboratory, so the next logical step would be to examine RSNO generation *in vivo*. A recently published study, using the assay developed in this work, has looked at the effect of intra-arterial infusion of low doses of authentic NO (*Rassaf*

et al. 2002a). They found that forearm blood flow, measured by plethysmography, increased for a more sustained period of time than would be predicted from the short half-life of NO *in vivo*. Moreover the vasodilatation was prolonged compared to the infusion of bradykinin and acetylcholine, which would lead to local NO release, and was associated with a mean increase of venous plasma RSNOs of 175% from a basal level of 40 ± 7 nmol. Subsequently the same group have shown that the vasodilatatory effect was reproduced by the infusion of S-nitrosoglutathione at concentrations which lead to the same increase in circulating RSNO levels (*Rassaf et al. 2002b*).

In conclusion it has been demonstrated that NO is capable of generating RSNOs in plasma, blood and *in vivo*. It has been shown from the second order kinetics of the reaction that a two-fold increase in NO production will lead to a four-fold increase in RSNO generation, and it has been shown that small incremental increases in venous S-nitrosothiol levels cause a significant increase in vasodilatation. Therefore in liver disease and during sepsis it may be predicted that the increase in NO production would be responsible for a significant increase in RSNO formation, which in turn may contribute to the vasodilatatory states seen in these conditons. This has, in part, been confirmed by the study of Ottesen et al. (*2001*) showing high levels in bile duct ligated rats, which were significantly increased by lipopolysaccharide.

In addition to examining the role of NO, peroxynitrite mediated generation of S-nitrosothiols was also examined. Peroxynitrite has vasorelaxant properties (*Villa et al. 1994*) and inhibits platelets in a thiol dependent fashion (*Moro et al. 1994*), and in buffer solutions is capable of inducing thiol nitrosation (*Mayer et al. 1998*). In this study peroxynitrite did lead to the generation S-nitrosthiols. The reaction kinetics were first order, and it occurred in the absence of oxygen. There was only a 0.25% conversion of peroxynitrite to RSNO, nevertheless in the context of liver disease with ongoing nitrosative stress these levels may be of relevance.

The data with SIN-1 is more difficult to interpret. It would have been predicted that the equimolar generation of NO and superoxide should favour the generation of peroxynitrite, and according to the previous experiments lead to a low production of S-nitrosothiols that obeyed first order kinetics. The data would suggest that in reality RSNO generation followed second order kinetics more closely. This finding of more efficient RSNO generation by SIN-1 than peroxynitrite is confirmed by a subsequent study (*Schrammel et al. 2003*), who suggested that thiols may compete with superoxide to form thiyl radicals, rather than generating peroxynitrite, which themselves react with NO to generate S-nitrosothiols, as illustrated below.

$$\mathbf{RSH + O_2^{*-} \rightarrow RSO^{*} + OH^{-}}$$

$$\mathbf{RSO^{*} + {}^{*}NO \rightarrow RSONO}$$

$$\mathbf{RSONO + R'SH \rightarrow RSOH + RSNO}$$

Since SIN-1 generates NO and O_2^- in equimolar concentrations, the finding that increasing concentrations of superoxide dismutase leads to increasing concentrations of RSNOs with the same concentration of SIN-1, would argue that peroxynitrite formation was a minor component in the reaction between SIN-1 and plasma.

In addition to exploring the formation of RSNOs in plasma, their stability was also examined. The half-life of SNO-albumin was significantly longer in plasma than that of the low molecular weight RSNO, S-nitrosoglutathione. One of the major factors responsible for the disappearance of RSNOs appears to be the presence of free thiol groups. Whereas thiol blockade by pre-treatment with NEM lead to a stabilisation of RSNOs, addition of high concentrations of glutathione significantly shortened the half-life of SNO-albumin in plasma. This could be related to a variety of factors, including transnitrosation reactions. Co-incubation of SNO-glutathione with plasma lead to the formation of SNO-albumin, however only 30% of the

original RSNO concentration was detected as SNO-albumin. This would suggest that in plasma the presence of free thiol groups leads to the release of NO as well as transnitrosation.

The importance of transnitrosation reactions in the biology of S-nitrosothiols was highlighted in a study by Jourd'heuil et al. (*2000*) who also examined the stability of S-nitrosothiols in plasma and whole blood. They confirmed that the major factor leading to the breakdown of SNO-glutathione was the presence of high molecular weight thiols i.e albumin. They were able to demonstrate that pretreatment with NEM and removal of the high molecular weight fraction (MW > 30,000) decreased the rate of decomposition by more than 80%. They also confirmed the work in this study that the major fate of SNO-glutathione was the formation of SNO-albumin. At variance with the study in this thesis, they found that up to 80% of the nitroso groups transnitrosated to albumin in plasma as opposed to 30% observed here; the reasons for this difference are unclear. Other factors purported to be of importance in *in vivo* degradation were excluded, including physiological levels of divalent metal cations and the generation of superoxide. Incubation of SNO-glutathione with whole blood showed that the presence of haemoglobin only had a relatively minor influence on the fate of low molecular weight S-nitrosothiols, as following a 30 minute incubation 60% of the original signal was detectable as SNO-albumin.

The transnitrosation reaction could be due to either the release of NO which then reacts with oxygen to form a nitrosating species, such as N_2O_3, that then generates a new RSNO, or via the direct transfer of NO^+ as illustrated below.

$$\mathbf{RSNO + R'S^- \leftrightarrow RS^- + R'SNO}$$

It would be straightforward to look at this by repeating the experiment demonstrating transnitrosation in a solution rendered anaerobic by sparging with helium, as also described.

Albumin thiol concentration is approximately 500 μM in plasma whereas glutathione is ~ 5 - 10 μM and cysteine 10 μM (*Stamler and Slivka 1996*). The presence of excess albumin in plasma would dictate that the above reaction equilibrium is shifted in the favour of an excess of SNO-albumin. This was confirmed by the demonstration that in healthy human plasma only high molecular weight S-nitrosothiols could be detected, and that the proportion of low molecular weight RSNOs formed by incubating plasma with authentic NO was less than 10%. It may therefore be that pharmacological manipulations that increase circulating low molecular weight thiols may result in the mobilisation of NO pools from high molecular weight thiols that may not be accessible otherwise.

The chemistry of transnitrosation may explain why administration of N-acetylcysteine as a bolus lead to a reduction in systemic vascular resistance in patients with fulminant hepatic failure and cirrhosis in the studies by Harrison (*1991*) and Jones (*1994*).

Lipoic acid is a low molecular weight thiol. Although the study looking at its administration in bile duct ligated rats concluded that the improvement in the hyperdynamic circulation was likely due to a decrease in nitric oxide synthase activity by comparison to controls, another mechanism is possible. The chronic administration of a low molecular weight thiol would be expected to shift the circulating pool away from SNO-albumin to SNO-lipoic acid. This compound has a longer half-life than SNO-glutathione and SNO-cysteine, and could theoretically be excreted in the urine, thereby, over a period of time reduce the total circulating pool of S-nitrosthiols. This hypothesis would be one direction for future work to continue on from this thesis.

7.4 SUMMARY

The work presented in this thesis has explored a completely novel area. Whereas previous work has looked at the role of oxidative and nitrosative stress on the progression of liver disease, this study has examined its effect on circulatory changes.

It has demonstrated that a product of lipid peroxidation, 8-isoprostaglandin $F_{2\alpha}$ is capable of increasing portal pressure, especially in rats with cirrhosis. This may explain why patients with cirrhosis are more prone to variceal bleeding when they are septic, due to surges in portal pressure.

This is the first demonstration that antioxidant treatment can prevent the development of the hyperdynamic circulation in rats with biliary cirrhosis, and shown that this is probably due to down-regulation of nitric oxide synthase acrivity.

Perhaps the most important work has been the development of an assay for plasma S-nitrosothiols. This assay has been subsequently used in several high quality publications, either as described or with minor modifications, which has lead to a greater understanding of nitric oxide chemistry in health and disease.

A great deal of questions remain unanswered, but on the basis of the work included in this thesis, there is a rationale to examine the role of long term antioxidant therapy in chronic liver disease, and its effects on many of the life threatening complications including variceal bleeding, ascites and hepatic encephalopathy.

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APPENDIX 1 – PUBLICATIONS ARISING FROM THIS THESIS

PEER REVIEWED JOURNAL ARTICLES

1) Formation of nanomolar concentrations of S-nitroso-albumin in human plasma by nitric oxide. **Free Radical Biology & Medicine.** 31(5):688-96, 2001. **Marley R,** Patel R, Orie N, Ceaser E, Darley-Usmar V, Moore K.

2) A chemiluminescense-based assay for S-nitrosoalbumin and other plasma S-nitrosothiols. **Free Radical Research.** 32(1): 1-9, 2000 **Marley R,** Feelisch M, Moore K.

3) Lipoic acid prevents development of the hyperdynamic circulation in anesthetized rats with biliary cirrhosis. **Hepatology.** 29(5): 1358-63, 1999. **Marley R,** Holt S, Fernando B, Harry D, Anand R, Goodier D, Davies S, Moore K.

4) Acute cholestasis-induced renal failure: effects of antioxidants and ligands for the thromboxane A2 receptor. **Kidney International.** 55(1):271-77, 1999. Holt S, **Marley R,** Fernando B, Anand R, Goodier D, Moore K.

5) Improvement in renal function in hepatorenal syndrome with N-acetylcysteine (letter). **Lancet.** 353(9149): 294-95, 1999. Holt S, Goodier D, **Marley R,** Patch D, Burroughs A, Fernando B, Goodier D, Moore K.

6) Increased sensitivity to endotoxaemia in the bile duct-ligated cirrhotic rat. **Hepatology.** 30(5): 1198-05, 1999. Harry D, Anand R, Holt S, Davies S, **Marley R,** Fernando B, Goodier D, Moore K.

7) N-acetylcysteine prevents development of the hyperdynamic circulation in the portal hypertensive rat. **Hepatology.** 28(3): 689-94, 1998. Fernando B, **Marley R,** Holt S, Anand R, Harry D, Sanderson P, Smith R, Hamilton G, Moore K.

8) 8-isoprostaglandin F2 alpha, a product of lipid peroxidation, increases portal pressure in normal and cirrhotic rats. **Gastroenterology.** 112(10): 208-13, 1997. **Marley R,** Harry D, Anand R, Fernando B, Davies S, Moore K.

PUBLISHED ABSTRACTS

1) **Marley, R.,** S. Holt and K. Moore. Increased formation of S-nitrosothiols in the acute bile duct ligated rat. *Hepatology* 30:A1647 (1999)

2) **Marley, R.,** D. Harry, B. Fernando, and K. Moore. Enhanced portal pressure response to ethanol in cirrhotic rats: the role of endothelin. *Hepatology* 24:A758 (1996).

3) **Marley, R.**, R. Anand, D. Harry, S. Holt, B. Fernando, and K. Moore. Increased sensitivity to endotoxemia in cirrhotic rats. ***Hepatology*** 26:A413 (1997)

4) **Marley, R.**, S. Holt, D. Goodier, D. Harry, B. Fernando, and K. Moore. Bile duct ligation and renal dysfunction in the rat. ***Hepatology*** 26:A75 (1997).

5) **Marley, R.,** B. Fernando, S. Holt, D. Harry, R. Anand and K. Moore. Lipoic acid prevents development of the hyperdynamic circulation in experimental cirrhosis. *Journal of Hepatology* 28:A76 (1998).

6) Holt, S., **R. Marley**, D. Goodier, D. Harry, B. Fernando, and K. Moore.. Oxidant stress and renal dysfunction in cholestasis. ***Gut*** 43:A153 (1998)

7) Holt, S., **R. Marley,** D. Goodier, B. Fernando, and K. Moore. Renal function is improved by N-acetylcysteine in patients with hepatorenal syndrome. ***Hepatology*** 28:A2003 (1998)

8) Holt S., **R. Marley,** D. Goodier, D. Harry, B. Fernando, and K. Moore. N-Acetylcysteine improves renal function in the acute bile duct ligated rat. ***Kidney International*** 55:2097 (1999)

9) Holt S., **R. Marley,** B. Fernando, D. Goodier, D. Patch, and K. Moore. N-Acetylcysteine improves renal function in the hepatorenal syndrome. ***Kidney International*** 55 :2574, (1999)

10) Harry, D., R. Anand, **R. Marley,** S. Holt, B. Fernando, and K. Moore.. Increased sensitivity to endotoxemia in cirrhosis. ***Gut*** 43: A152 (1998)

11) Holt, S., **R. Marley,** B. Fernando, D. Harry, R. Anand, D. Goodier, L.J. Roberts, and K. Moore. Renal dysfunction and oxidative stress following bile duct ligation. ***Journal of the American Society of Nephrology*** 8:A2737 (1997)

12) Fernando, B., **R. Marley**, S. Holt, R. Anand, D. Harry, G. Hamilton, and K. Moore. Oxidant stress and the hyperdynamic circulation of portal hypertension. ***Hepatology*** 26: A925 (1997)

13) Harry, D., R. Anand, **R. Marley,** S. Holt, B. Fernando, and K. Moore.. Oxidative response to endotoxemia in cirrhosis. ***Hepatology*** 28: A1866 (1998)

APPENDIX 2 – ABBREVIATIONS

8-iso-$PGF_{2\alpha}$	8-isoprostaglandin $F_{2\alpha}$
AE-1	anion exchange protein 1
ANOVA	analysis of variances
BDL	bile duct ligated
BH_4	tetrahydrobiopterin
BSTFA	bis-silyltrimethylfluouroacetamide
CCl_4	carbon tetrachloride
CI	cardiac index
COX	cyclooxygenase
DIPEA	diisopropylethylamine
DNA	deoxyribonucleic acid
DTPA	diethylenetriamine pentaacetic acid
EDTA	ethylene diamine tetraacetic acid
EPR	electron paramagnetic resonance
ET	endothelin
FAD	flavine adenine dinucleotide
FMN	flavine mononucleotide
GCMS	gas chromatography mass spectroscopy
GSH	reduced glutathione
GTP	guanine triphosphate
HAK	4-hydroxy-2,3-alkenal
HNE	4-hydroxy nonenal
HPLC	high purification liquid chromatography
IL-	interleukin
IFN-γ	interferon gamma
LDL	low density lipoprotein

LFT	liver function test
LMW	low molecular weight
LPS	lipopolysaccharide
MAP	mean arterial pressure
MDA	malondialdehyde
mRNA	messenger ribonucleic acid
MWCO	molecular weight cut off
NAC	n-acetylcysteine
NADP	nicotinamide adenine dinucleotide phosphate
NFκB	nuclear factor kappa B
NEM	n-ethylmaleimide
NOS	nitric oxide synthase
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase
NS	not significant
PFBR	pentafluorobenzylbromide
PBS	phosphate buffered saline
PP	portal pressure
RNOS	reactive nitrogen species
ROS	reactive oxygen species
RSNO	S-nitrosothiol
SEM	standard error of the mean
SIN-1	sydnonimine
SNO-albumin	S-nitrosoalbumin
SNO-cysteine	S-nitrosocysteine
SNO-glutathione	S-nitrosoglutathione

SNO-Hb	S-nitrososhaemoglobin
SOD	superoxide dismutase
SVRI	systemic vascular resistance index
TBARS	thiobarbituric acid reacting substances
TNF-α	tumour necrosis factor alpha
TLC	thin layer chromatography
TP	thromboxane

APPENDIX 3 – CHILDS-PUGH SCORE

	Points scored for increasing abnormality		
Clinical and biochemical measurements	1	2	3
Encephalopathy	None	Grade 1 and 2	Grade 3 and 4
Ascites	Absent	Slight	Moderate
Bilirubin (mg 100ml)	1 - 2	2 - 3	>3
Albumin (g l)	>35	28 - 35	<28
Prothrombin (s prolonged)	>35	28 - 35	<28
For primary biliary cirrhosis Bilirubin (mg 100ml)	1 - 4	4 - 10	>10

Childs Pugh grading of the severity of liver disease (*Pugh et al. 1973*)

Grade A score 5 6

Grade B score 7 9

Grade C score 10 - 15

APPENDIX 4 - SOLUTIONS USED FOR STELLATE CELL ISOLATION

A) Perfusion Buffer

In 1 litre double distilled water (DDW)

- NaCl - 8g
- KCl - 0.4g
- Na_2HPO_4 - 0.19g
- KH_2PO - 0.06g
- + 5 ml 7.5% $NaHCO_3$
- + 10 ml 1M HEPES
- + 10 ml penicillin G (10,000U / ml)

bubbled with 95% O_2 / 5% CO_2

B) Pronase solutions

High pronase - dissolve 260 mg pronase (Boehringer. Mannheim catalogue # 1459643) in 100 ml HAMS / Dulbecco MEM solution

Low pronase - dissolve 27.5 mg pronase in 50 ml HAMS / Dulbecco MEM solution

C) Nycodenz gradient tubes

Stock solutions - made up to 1 litre with DDW

10X GBSS (+ NaCl)

- KCl - 3.7g
- KH_2PO_4 - 0.3g
- NaCl - 80g
- $NaHCO_3$ - 2.27g
- Na_2HPO_4 - 1.2g
- D-glucose - 10g

10X GBSS (- NaCl)

- KCl - 3.7g
- KH_2PO_4 - 0.3g
- $NaHCO_3$ - 2.27g
- Na_2HPO_4 - 1.2g
- D-glucose - 10g

10X $CaCl_2$

- $CaCl_2$ (anhydrous)- 1.7g

10X $MgCl_2$

- $MgCl_2$ (anhydrous) - 0.98g
- $MgSO_4$ (anhydrous) - 0.34g
- $MgSO_4.7H_2O$ - 0.7g

To prepare gradient tubes make up

50 ml 1X GBSS (+ NaCl)

- 5 ml 10X GBSS (+)
- 5 ml 10X $CaCl_2$
- 5ml 10X $MgCl_2$
- 35 ml DDW

50 ml 1X GBSS (- NaCl)

- 5ml 10X GBSS (-)
- 5 ml 10X $CaCl_2$
- 5ml 10X $MgCl_2$
- 35 ml DDW

pH both solutions to between 7.0 - 7.4

Make up 28.7% (wt/vol) nycodenz in 1X GBSS (-) (= 7.2g / 25 ml)

Dilute 28.7% to 15.6% and 8.2% in 1X GBSS (+)

Check optical density with refractometer.